Peripheral neuropathy in type 1 and type 2 diabetes – screening of vibrotactile sense and proteomics in human nerve biopsies

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Peripheral neuropathy in type 1 and type 2 diabetes

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– screening of vibrotactile sense and proteomics in human nerve biopsies

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Title and subtitle: Peripheral neuropathy in type 1 and type 2 diabetes – screening of vibrotactile sense and proteomics in human nerve biopsies

Abstract

Background: Diabetic peripheral neuropathy (DPN) is a common complication of type 1 diabetes (T1D) and type 2 diabetes (T2D). DPN may lead to pain, impaired quality of life and is the main risk factor for foot ulcer development. Finding DPN at early stages can be difficult using conventional screening methods. The pathogenesis of DPN is multifactorial and depends on several co-existing pathways. However, DPN does not present in the same way in T1D and T2D. Improvement of metabolic control in T1D may reduce DPN, while improved metabolic control in T2D only has scarce, if any, effect on DPN. **Aims:** The overall aim was to approach DPN from two new perspectives. In the first part, the aim was to screen for DPN in children and adolescents with T1D using multi-frequency vibrometry (MFV). In the second part, the aim was to describe the proteome of the posterior interosseous nerve (PIN), and to look for proteins that were differentially expressed, in subjects with T1D, T2D and healthy controls. **Methods:** In the first part, children and adolescents with T1D, aged 8-18 years, were invited for screening. Examinations with MFV and monofilaments, examining perception to light touch, were conducted at baseline at two sites on the hand and at two sites on the foot. Analyses were carried out to look for associations between impaired vibrotactile sense and characteristics, like age, disease duration, HbA1c, treatment and the presence of autoantibodies to glutamic acid decarboxylase 65 (GAD65) and/or insulinoma associated protein 2 (IA-2). Participants were invited to take part in a follow-up, to study potential alterations of vibrotactile sense over time.

In the second part, subjects with T1D and T2D, as well as, healthy controls were recruited from two previous cohorts. Biopsies of PIN were harvested under local or regional anaesthesia and stored in a freezer at -80°C until analysis. PINs were analysed using liquid chromatography tandem mass spectrometry. Nerve conduction studies (NCSs) of ulnar, peroneal and sural nerves were conducted in all subjects. Quantitative mass spectrometry data was analysed to look for associations with nerve conduction. **Results:** In the first part, 72 children and adolescents with T1D were examined with MFV at baseline, and 37 subjects accepted follow-up after a median of 30 months. At baseline, 13 out of 72 subjects (18%) showed signs of impaired vibrotactile sense on at least one site on the foot. At follow-up, 3 out of 37 subjects (8%) showed signs of impaired vibrotactile sense, but one of those three had normal MFV examination at baseline. Impaired vibrotactile sense was not associated with age, disease duration or HbA1c at baseline or follow-up. Neither was impaired sense associated with autoantibodies to GAD65 and/or IA-2. At baseline, impaired vibrotactile sense was more common among subjects receiving insulin as multiple daily injections compared to subjects receiving insulin through continuous subcutaneous insulin infusion. In the second part, I identified and quantified 2617 different proteins in PIN from subjects with T1D, T2D and healthy controls using quantitative mass spectrometry. In a first study, on nine subjects with T2D and six healthy, gender and age matched controls, I did not see any statistically significant difference in protein expression. In a subsequent study on 56 subjects (T1D: n = 9, T2D: n = 24, healthy controls: n = 23), a total of 32 heat shock proteins (HSPs) were identified and quantified. However, protein expressions of HSPs did not differ significantly between T1D, T2D and healthy controls, and no correlations were seen to nerve function of the ulnar nerve. **Conclusions:** Firstly, MFV can be used to screen for impaired vibrotactile sense, mirroring DPN, among children and adolescents with T1D, but the vibrotactile sense might change and even improve over time, unrelated to HbA1c. Secondly, quantitative mass spectrometry can be used to describe protein expressions in PIN in subjects with T1D, T2D and healthy controls. However, in order to determine differences in protein expressions of interest to DPN, between groups, it is likely that more severe types of DPN need to be studied. Alternatively, larger study groups may be needed. The present thesis suggests two novel methods to 1. look for early signs of DPN in paediatric subjects with T1D, and 2. present quantitative proteomics from whole nerve biopsies of subjects with T1D and T2D. The first technique can be used in future studies to screen for DPN and the second may aid in future search for biomarkers of DPN in T1D and T2D.

Key words: type 1 diabetes, type 2 diabetes, peripheral neuropathy, vibrotactile sense, multi-frequency vibrometry, nerve biopsy, proteomic

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Cover photo by Erik Ising. Protein network is adapted from String database v11[1]

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MADE IN SWEDEN

To my family

Table of Contents

Abstract

Background

Diabetic peripheral neuropathy (DPN) is a common complication of type 1 diabetes (T1D) and type 2 diabetes (T2D). DPN may lead to pain, impaired quality of life and is the main risk factor for foot ulcer development. Finding DPN at early stages can be difficult using conventional screening methods. The pathogenesis of DPN is multifactorial and depends on several co-existing pathways. However, DPN does not present in the same way in T1D and T2D. Improvement of metabolic control in T1D may reduce DPN, while improved metabolic control in T2D only has scarce, if any, effect on DPN.

Aims

The overall aim was to approach DPN from two new perspectives. In the first part, the aim was to screen for DPN in children and adolescents with T1D using multi-frequency vibrometry (MFV). In the second part, the aim was to describe the proteome of the posterior interosseous nerve (PIN), and to look for proteins that were differentially expressed, in subjects with T1D, T2D and healthy controls.

Methods

In the first part, children and adolescents with T1D, aged 8-18 years, were invited for screening. Examinations with MFV and monofilaments, examining perception to light touch, were conducted at baseline at two sites on the hand and at two sites on the foot. Analyses were carried out to look for associations between impaired vibrotactile sense and characteristics, like age, disease duration, HbA1c, treatment and the presence of autoantibodies to glutamic acid decarboxylase 65 (GAD65) and/or insulinoma associated protein 2 (IA-2). Participants were invited to take part in a follow-up, to study potential alterations of vibrotactile sense over time.

In the second part, subjects with T1D and T2D, as well as, healthy controls were recruited from two previous cohorts. Biopsies of PIN were harvested under local or regional anaesthesia and stored in a freezer at -80°C until analysis. PINs were analysed using liquid chromatography tandem mass spectrometry. Nerve conduction studies (NCSs) of ulnar, peroneal and sural nerves were conducted in all subjects. Quantitative mass spectrometry data was analysed to look for associations with nerve conduction.

Results

In the first part, 72 children and adolescents with T1D were examined with MFV at baseline, and 37 subjects accepted follow-up after a median of 30 months. At baseline, 13 out of 72 subjects (18%) showed signs of impaired vibrotactile sense on at least one site on the foot. At follow-up, 3 out of 37 subjects (8%) showed signs of impaired vibrotactile sense, but one of those three had normal MFV examination

at baseline. Impaired vibrotactile sense was not associated with age, disease duration or HbA1c at baseline or follow-up. Neither was impaired sense associated with autoantibodies to GAD65 and/or IA-2. At baseline, impaired vibrotactile sense was more common among subjects receiving insulin as multiple daily injections compared to subjects receiving insulin through continuous subcutaneous insulin infusion.

In the second part, I identified and quantified 2617 different proteins in PIN from subjects with T1D, T2D and healthy controls using quantitative mass spectrometry. In a first study, on nine subjects with T2D and six healthy, gender and age matched controls, I did not see any statistically significant difference in protein expression. In a subsequent study on 56 subjects (T1D: n = 9, T2D: n = 24, healthy controls: n = 23), a total of 32 heat shock proteins (HSPs) were identified and quantified. However, protein expressions of HSPs did not differ significantly between T1D, T2D and healthy controls, and no correlations were seen to nerve function of the ulnar nerve.

Conclusions

Firstly, MFV can be used to screen for impaired vibrotactile sense, mirroring DPN, among children and adolescents with T1D, but the vibrotactile sense might change and even improve over time, unrelated to HbA1c. Secondly, quantitative mass spectrometry can be used to describe protein expressions in PIN in subjects with T1D, T2D and healthy controls. However, in order to determine differences in protein expressions of interest to DPN, between groups, it is likely that more severe types of DPN need to be studied. Alternatively, larger study groups may be needed.

The present thesis suggests two novel methods to 1. look for early signs of DPN in paediatric subjects with T1D, and 2. present quantitative proteomics from whole nerve biopsies of subjects with T1D and T2D. The first technique can be used in future studies to screen for DPN and the second may aid in future search for biomarkers of DPN in T1D and T2D.

Abbreviations

ADA	American Diabetes Association
AGE	advanced glycation end-products
CGM	continuous glucose monitor
CNS	central nervous system
CSII	continuous subcutaneous insulin infusion
CTS	carpal tunnel syndrome
DAG	diacylglycerol
DCCT	Diabetes Control and Complications Trial
DNA	deoxyribonucleic acid
DM	diabetes mellitus
DSPN	diabetic symmetrical length-dependent sensorimotor polyneuropathy
DPN	diabetic peripheral neuropathy
DRG	dorsal root ganglion
EDIC	Epidemiology of Diabetes Interventions and Complications
ELISA	enzyme-linked immunosorbent assay
GAD65	glutamic acid decarboxylase-65
GlcNac	uridine 5-diphosphate-N-acetylglucosamine
GWAS	genome wide association studies
HAVS	hand-arm vibration syndrom
HLA	human leukocyte antigen
HSF1	heat shock factor 1
HSP	heat shock protein
HSR	heat shock response
IA-2	insulinoma-associated protein 2
IAA	insulin autoantibody
ISPAD	International Society for Pediatric and Adolescent Diabetes
JNK	jun N-terminal kinase
LC-MS/MS	liquid chromatography tandem mass spectrometry

LTP	light touch perception
MAPK	mitogen-activated protein kinase
MDI	multiple daily injections of insulin
MHC	major histocompatibility complex
MODY	maturity onset diabetes of the young
NCS	nerve conduction study
NF-κB	nuclear factor kappa B
OGTT	oral glucose tolerance test
PARP	poly(ADP-ribose) polymerase
PKC	protein kinase C
PNS	peripheral nervous system
PIN	posterior interosseous nerve
RAGE	AGE receptor
RNA	ribonucleic acid
ROS	reactive oxygen species
SD	standard deviation
SNP	single nucleotide polymorphism
T1D	type 1 diabetes
T2D	type 2 diabetes
TGF-β	transforming growth factor-beta
VEGF	vascular endothelial growth factor
VPT	vibration perception threshold
Zn-8	zinc transporter 8

List of papers

This thesis is based on the following papers, which will hereafter be referred to by their Roman numerals.

Paper I: Impaired vibrotactile sense in children and adolescents with type 1 diabetes - Signs of peripheral neuropathy

Erik Ising, Lars B. Dahlin, Helena Elding Larsson

PLoS ONE. 2018; 13(4): e0196243.

Paper II: Impaired vibrotactile sense showed no association with insulinoma associated protein 2 and glutamic acid decarboxylase autoantibodies in paediatric type 1 diabetes

Erik Ising, Lars B. Dahlin, Helena Elding Larsson.

Acta Paediatrica. 2020; 109(10):2160-2161.

Paper III: Vibrotactile sense might improve over time in paediatric subjects with type 1 diabetes – a mid-term follow-up using multifrequency vibrometry

Erik Ising, Linnéa Ekman, Helena Elding Larsson, Lars B. Dahlin.

Acta Paediatrica. 2022; 111:411–417. Epub 2021.

Paper IV: Quantitative proteomic analysis of human peripheral nerves from subjects with type 2 diabetes.

Erik Ising, Emma Åhrman, Niels O. B. Thomsen, Karl-Fredrik Eriksson, Johan Malmström, Lars B. Dahlin.

Diabetic Medicine. 2021; 38(11):e14658.

Paper V: Expression of heat shock proteins in the posterior interosseous nerve among subjects with type 1 and type 2 diabetes compared to healthy controls

Erik Ising, Emma Åhrman, Niels O. B. Thomsen, Anna Åkesson, Johan Malmström, Lars B. Dahlin.

Manuscript.

Thesis at a glance

Paper I: Impaired vibrotactile sense in children and adolescents with type 1 diabetes - Signs of peripheral neuropathy

Aim: To study the presence of impaired vibrotactile sense among children and adolescents with type 1 diabetes (T1D).

Methods: In total 72 children and adolescents were examined for vibrotactile sense, using multi-frequency vibrometry (MFV), and light touch perception (LTP), using Semmes-Weinstein's monofilaments, for signs of diabetic peripheral neuropathy (DPN). Both hands and feet were examined.

Results and conclusions: Impaired vibrotactile sense was seen in 13 out of 72 subjects. No subjects presented with impaired LTP. Impaired vibrotactile sense was not associated with age, gender, HbA1c or height. However, subjects receiving insulin through multiple daily injections (MDI) had a higher proportion of impaired vibrotactile sense compared to subjects receiving insulin through continuous subcutaneous insulin infusion (CSII).

Paper II: Impaired vibrotactile sense showed no association with insulinoma associated protein 2 and glutamic acid decarboxylase autoantibodies in paediatric type 1 diabetes

Aim: To study associations between autoantibodies to glutamic acid decarboxylase 65 (GAD65) and insulinoma associated protein 2 (IA-2) and impaired vibrotactile sense in paediatric subjects with T1D.

Methods: Data on GAD65 and IA-2 positivity at diagnosis of T1D was present in a total of 70 out of 72 participants from Paper I, and hence 70 subjects were included for statistical analyses. Analyses were performed to study if impaired vibrotactile sense was associated with autoantibody positivity from the time of clinical T1D diagnosis.

Results and conclusions: No associations were seen between impaired vibrotactile sense and the presence of autoantibodies to GAD65 ($p = 0.74$), IA-2 ($p = 1.0$) or both ($p = 1.0$). Thus, our data does not support an association between impaired vibrotactile sense and autoantibodies against GAD65 and/or IA-2.

Paper III: Vibrotactile sense might improve over time in paediatric subjects with type 1 diabetes - A mid-term follow-up using multifrequency vibrometry

Aim: To study the variability of vibrotactile sense in paediatric subjects with T1D

Methods: All participants from Paper I was invited to participate in a follow-up study. A follow-up examination of the vibrotactile sense was conducted in 37 children and adolescents in median 30 months from baseline.

Results and conclusions: Vibrotactile perception improved at low frequencies, especially at 16 Hz, on the foot. No associations were seen between vibrotactile sense and HbA1c. The results suggests that vibrotactile sense might fluctuate over time in T1D irrespective of metabolic control.

Paper IV: Quantitative proteomic analysis of human peripheral nerves from subjects with type 2 diabetes

Aim: To describe the proteome of the posterior interosseous nerve (PIN) in subjects with type 2 diabetes (T2D) and healthy controls.

Methods: Nine subjects with T2D and six healthy controls were recruited from two previous cohorts. All subjects had already undergone PIN biopsy and electrophysiology examinations. PINs were analysed using quantitative mass spectrometry. Differences in protein expression between subjects with T2D and controls were studied.

Results and conclusions: In total, 2617 different proteins were identified and quantified across all samples. A linear regression model did not reveal any differences in protein expression between groups. The cluster analysis revealed three significant clusters of similar proteins. This study concludes that quantitative mass spectrometry can be used to describe the proteome of PIN.

Paper V: Expression of heat shock proteins in the posterior interosseous nerve among subjects with type 1 and type 2 diabetes compared to healthy controls

Aim: To describe the quantitative expression of heat shock proteins (HSPs) among subjects with T1D, T2D and healthy controls.

Methods: PINs from 56 subjects (T1D: n = 9; T2D: n = 24; healthy controls: n = 23) were studied using quantitative mass spectrometry. Differences in protein expression between groups were analysed using linear regression, and protein intensities were correlated to electrophysiology parameters.

Results and conclusions: A total of 32 different HSPs were identified in PIN across all samples. No clear differences in protein expressions of HSPs were seen between groups.

Introduction

It is estimated that 463 million people, between 20 and 79 years of age, are living with diabetes worldwide (1). This accounts for approximately ten percent of the world population in that age group, making diabetes a global epidemic. The total number is predicted to rise over the years to come (1), presenting the world with significant challenges regarding disease management, and management of the complications from diabetes.

The focus of this doctoral thesis is DPN, often presenting with impaired sense in a symmetrical stocking and glove pattern to the distalmost parts of the lower, but also the upper, extremities. DPN is a potentially fatal complication to both T1D and T2D, that may lead to severe foot ulcers, which can significantly impair the quality of life for the affected person (1, 2). Furthermore, taking care of DPN and foot ulcers also incurs large costs for the health care system worldwide (3-5).

As of today, there is no cure for DPN, and the pathogenesis is not fully understood. Although several co-existing pathways contributing to the pathogenesis of DPN have been described, there is still a need to approach this well-known, and severe, complication from new perspectives.

In this thesis, I am presenting novel aspects on DPN and aim to address the complication from new perspectives. I suggest that impaired vibrotactile sense, a sign of DPN, is present among some paediatric subjects with T1D and can be screened for using MFV. Furthermore, I present for the first time, to the best of my knowledge, quantitative mass spectrometry data from completely unique human peripheral nerve biopsies (i.e. the posterior interosseous nerve) from subjects with T1D, T2D and healthy controls. I strongly believe that quantitative mass spectrometry from nerve biopsies will have the possibility to aid in future searches for biomarkers and potential drug agents for DPN.

Background

Diabetes mellitus

Diabetes mellitus (DM) is not one but a range of diseases affecting the body's ability to regulate blood glucose levels, leading to hyperglycaemia with consecutive metabolic changes and adaptations and subsequently complications to the diseases (6, 7). The World Health Organization defines DM by one of four diagnostic measures; a fasting venous plasma blood glucose of ≥ 7.0 mmol/L, a venous plasma blood glucose of ≥ 11.1 mmol/L at 2 hours after an oral intake of 75 grams of glucose [oral glucose tolerance test (OGTT)], a random venous plasma blood glucose of ≥ 11.1 mmol/L with concurrent signs or symptoms of DM or a HbA1c value of ≥ 48 mmol/mol (6). An HbA1c value of ≥ 48 mmol/mol is, however, not enough to diagnose diabetes in children and adolescents (8). The typical symptoms of disease onset in DM, like thirst, polyuria, weight loss and blurred vision, are most common at onset of T1D and not as frequent at onset of T2D (6). However, T2D in youth commonly presents with typical symptoms, and diabetic ketoacidosis at presentation of T2D has been shown in around one tenth of the subjects (9). The main reason for the lack of typical symptoms when T2D presents in adults is that T2D development may occur for years before the disease is diagnosed, while the development of symptomatic T1D is usually much quicker (6). The different ways the two types of DM arise demonstrate major dissimilarities in the aetiology of the diseases.

Types of diabetes mellitus

DM can either be caused by an absolute, or a relative, insulin deficiency and the aetiology of the deficiency denotes the type, i.e. T1D, T2D and other specific types (10).

T1D is typically caused by an autoimmune response and destruction of pancreatic beta cells, leading to a successive insulin depletion until the insulin deficiency is absolute, while T2D, the most common type of diabetes, leads to an impaired glucose metabolism accompanied with insulin resistance (10, 11). Besides T1D and T2D, there are other, less common types of DM, for example caused by genetic defects of beta cell function, i.e. maturity onset diabetes of the young (MODY), or caused by infections, genetical syndromes, drug treatment etc. (10, 12). This thesis

will solely focus on T1D and T2D, and complications arising from these diseases. Hereafter, the term DM will be used synonymously with diabetes when referring to both T1D and T2D.

T1D is most commonly diagnosed before the age of 30 and has historically been described as DM of the young, but can present in all ages (6). T2D is more common among older adults, but the prevalence is rising in younger adults and also in children and adolescents, mainly because of its connection to the metabolic syndrome, i.e. insulin resistance syndrome, and obesity (12-14). However, of all people suffering from diabetes 90-95% are diagnosed with T2D, while in paediatric subjects with diabetes the vast majority are suffering from T1D (6, 15, 16). Both T1D and T2D have the ability to affect several tissues of the body and complications due to micro- and macroangiopathies are common (2, 17). Among the most common microangiopathic complications are diabetic nephropathy and diabetic retinopathy, but also diabetic neuropathies of different types (2, 17). Although the aetiological nature of diabetic neuropathies is not solely angiopathic, as will be discussed further on, the nomenclature "microangiopathy" is commonly used.

Complications to T1D and T2D

Both T1D and T2D are associated with significant risks of developing complications from the diseases, leading to both impaired quality of life for the subject, as well as large monetary costs for the health care system and also to society, due to inability to work among some affected subjects (3-5). The aetiology of complications in T1D and T2D is multifactorial, but hyperglycaemia plays an essential role, and many complications deteriorate with increasing disease duration (18-22). In general, complications to T1D and T2D can be divided into macrovascular and microvascular (23, 24). Macrovascular complications present with cardiac diseases, such as coronary artery disease, cerebral ischemia, i.e. ischemic stroke, and peripheral artery disease (23, 25). Microvascular complications include retinopathy, nephropathy and neuropathy, where the latter can be subdivided into autonomic and peripheral neuropathies (2, 24)

Autoimmunity in T1D

An autoimmune response to pancreatic beta cells ultimately leads to symptomatic T1D. Usually, months to years before symptoms appear, autoantibodies directed to beta cells may form (7, 10). The first autoantibodies to form are commonly autoantibodies to GAD65 or insulin-antigen antibody (IAA) (7, 10). Later in the course of T1D development, autoantibodies targeting IA-2 or zinc transporter 8 (Zn-8) may be present (7, 10). What causes the formation of autoantibodies to beta cells is not clear, but individuals of specific HLA genotypes have a higher risk of forming two or more autoantibodies to beta cells (7). With an increasing number of different

autoantibodies, the higher the risk is of developing symptomatic T1D (7, 26). However, autoantibodies may also vanish and be absent at clinical onset of T1D, but in such cases, and without knowledge that autoantibodies have previously been present in the subject, other types of diabetes should be considered (27).

A possible link between the presence of GAD65 autoantibodies and DPN in T1D has been suggested, but reports are contradictory and the used methods vary extensively (28-33). The connection between GAD65 and peripheral nerves is nevertheless intriguing; GAD65 is not only present in the pancreatic insulin producing beta cells, but also in the nervous system, where it is involved in the formation of the inhibitory neurotransmitter GABA (29, 34, 35).

Genetics of T1D and T2D

The pathogeneses of T1D and T2D are multifactorial, but genetic factors play a role in disease development (7, 11, 26, 36). Thus, the genetics of T1D and T2D are not dependant on single genes.

Approximately 50% of the genetic susceptibility to develop T1D is associated with human leukocyte antigen (HLA) class II, also known as major histocompatibility complex (MHC) class II (37, 38). More specifically, HLA class II are molecules on the surface of antigen presenting cells, like B-cells, dendritic cells, macrophages and activated T-cells, that present antigen to T-cells (i.e. the T-cell receptor on CD4+ T-cells are presented to an antigen by antigen presenting cells), with a subsequent immunological response (39). The HLA class II molecule consists of two subunits, an alpha-chain (with two domains; $\alpha 2$ and $\alpha 2$) and a beta-chain (also consisting of two domains; $\beta 1$ and $\beta 2$), encoded from genes located on chromosome 6. The region on chromosome 6 has three distinct loci, i.e. DP, DQ and DR, each containing genes that code for different subunits. Each gene exists in pairs, one on each chromosome, inherited from the mother and the father, respectively. Genes are expressed differently in different people due to variants in the genetic code. Such variants are dependent on different alleles which together form a haplotype. For example, this means that a person inherits one haplotype, comprising two alleles, of the DQ gene from their mother and one haplotype from their father and together these haplotypes denotes the genotype. The risk of developing T1D has been shown to be higher among people with HLA-DR3-DQ2 or HLA-DR4-DQ8, while having HLA-DQ6 has been shown to be, in part, protective against T1D (26, 37, 38, 40-42). Associations between HLA haplotype and the first autoantibodies to present in T1D have been shown. For example, subjects homozygote, meaning they have the same haplotype on both chromosomes, for HLA DR3-DQ2 tend to present with autoantibodies to GAD65 first (43), while IAA as first autoantibody is more common among subjects heterozygote for HLA DR3-DQ2/DR4-DQ8 (44).

From genome wide association studies (GWAS), single nucleotide polymorphisms (SNPs) of several genes have been shown to be associated with risk of developing T2D (11, 45, 46). However, carrying these risk alleles does not guarantee T2D development. In fact, the majority of people without T2D have risk alleles for T2D, but never develop the disease (11, 47). In conclusion, the genetic susceptibility to T2D must be accompanied by environmental and epigenetic factors.

The peripheral nervous system

The nervous system is divided into the central nervous system (CNS), comprising the brain and the spinal cord, and the peripheral nervous system (PNS). In the PNS the autonomic nervous system is one of the two motor parts, alongside the somatic motor system (48). The autonomic nervous system is subdivided into the sympathetic, parasympathetic and enteric parts, innervating smooth muscles, cardiac muscle and glands. The somatic motor system of the PNS innervates skeletal muscles with signals from the brain and spinal cord, while the sensory part of the PNS picks up external stimuli from sensory receptors, such as vibration sensitive mechanoreceptors, like Pacinian and Meissner corpuscles, and transmits them to the brain for processing (49-51).

Anatomy of the peripheral nerve

A neuron comprises a cell body and projections, i.e. axons and dendrites. These projections connects the neuron to other neurons, through synapses, or muscles, through motor end plates, to/from which they send or receive transmission signals. The spinal nerves from the spinal cord to the periphery of the body contain both efferent and afferent nerve fibres carrying neural impulses in the direction from and towards the CNS, respectively. The efferent fibres have axonal projections connected to a cell body located in the grey matter of the spinal cord, while the cell bodies of afferent nerve fibres are found outside of the spinal cord in the dorsal root ganglion (DRG).

The peripheral nerve consists of several nerve fascicles, surrounded by a perineurium, and blood vessels embedded in a loose layer of connective tissue called epineurium. Each fascicle contains a bundle of both sensory and motor axons. The axons are surrounded by supporting glial cells, of whom the Schwann cells are the most common. Schwann cells are either myelinating or non-myelinating. The myelinating Schwann cells create a sheet of myelin around single axons providing an environment enabling saltatory transmission of the action potential, resulting in faster neural transmission than in non-myelinated nerve fibres. The saltatory movement of action potentials is possible because the myelin sheets do not overlap.

Thus, an unmyelinated portion of the axon is created where two myelin sheets approach each other, i.e. the nodes of Ranvier. In non-myelinated nerve fibres, a Schwann cell is related at each segment of the nerve to several axons. Nerve fibres do not only differ in terms of myelination or not, but also in diameter. Thin unmyelinated nerve fibres, called C-fibres, are responsible for pain- and heat transmission from the periphery to the spinal cord. The nerve fibres transmitting signals from cutaneous mechanoreceptors, like Meissner's and Pacinian corpuscles (further described below), are named Aβ fibres and are, compared to C-fibres, thicker and myelinated.

Posterior interosseous nerve

Distal to the supinator muscle in the forearm, the continuation of the radial nerve forms the posterior interosseous nerve (PIN); a sensory and motor nerve providing proprioception to the distal radio-ulnar articulate and innervating several muscles in the posterior compartment of the forearm (52, 53). The distalmost part of PIN is situated alongside the posterior interosseous artery and can be quite easily harvested (54). This part of PIN has been studied regarding DPN in both T1D and T2D and has been proposed a suitable biopsy target instead of using sural nerves for studies on DPN (54-57).

Diabetic peripheral neuropathy

Diabetic peripheral neuropathy, or distal symmetric polyneuropathy, is the most common type of diabetic neuropathy (17). It usually presents with decreased sensation and pain in a stocking and glove pattern, meaning that the distalmost parts of the body are affected symmetrically, i.e. the distal parts of the lower extremities and occasionally also the upper extremities are affected first (2, 17, 58, 59). DPN is typically subclinical in the beginning and is common among adults with T1D and T2D, but DPN has also been shown to be present among children and adolescents with T1D (29, 59-62). Electrophysiology, i.e. nerve conduction studies (NCSs), on children and adolescents with T1D have proposed abnormalities in 28-58 % of examined subjects, providing evidence of a widespread complication early in the course of diabetes (63).

Definition

Several different definitions of DPN have been proposed. Boulton et al. 1998, in collaboration with NeuroDiab, a subgroup of the European Association for the Study of Diabetes, suggested a simple definition of DPN as "the presence of

symptoms and/or signs of peripheral nerve dysfunction in people with diabetes, after exclusion of other causes" (64). This definition is useful, but also too diffuse for research purposes. Tesfaye et al. 2010, on behalf of the Toronto Diabetic Neuropathy Expert Group, engaged in updating definitions and diagnostic criteria of DPN (65). Tesfaye et al. 2010 describes the typical DPN as "a chronic, symmetrical, length-dependent sensorimotor polyneuropathy (DSPN)" which can be either confirmed or subclinical (65). The confirmed DSPN must include an abnormal NCS, or an abnormal validated measure of small nerve fibre neuropathy, as well as sign(s) and/or symptom(s) of DSPN (65). Conversely, the subclinical DSPN must have an abnormal NCS or an abnormal validated small nerve fibre neuropathy examination, in the absence of sign(s) and/or symptom(s) of DSPN (65). Moreover, Tesfaye et al. 2010 describes possible DSPN as having sign(s) and/or symptom(s) typical for DSPN, and probable DSPN as having at least two of the following: "neuropathic symptoms, decreased distal sensation, or unequivocally decreased or absent ankle reflexes" (65).

For reasons of simplicity, and in order to reduce the risk of confusion, the term DSPN will hereafter be used synonymously with DPN. The above stated definitions of confirmed DPN are useful when planning, conducting and comparing research studies, but less important in the clinical setting. In the clinic, the presence, confirmed or suspected, of DPN should be highlighted and measures should be taken to minimize the risks of DPN.

Detection and screening

In the clinical setting, a NCS is seldom conducted in order to diagnose DPN, but can be used in unclear cases in order to confirm the diagnosis (24, 59). The International Society for Pediatric and Adolescent Diabetes (ISPAD) and the American Diabetes association (ADA) both suggest an annual screening of DPN in children and adolescents with diabetes (66, 67). ISPAD recommends starting with DPN screening in paediatric subjects from 11 years of age, having had diabetes for 2 to 5 years, while ADA recommends starting screening for DPN at the age of 10 years, and with 5 years of diabetes duration (66, 67). Furthermore, both ISPAD and ADA recommend screening of both small fibre (i.e. temperature and pin-prick sensation) and large fibre neuropathy (sensation to vibration and light touch), together with a clinical examination (66, 67). For adults with diabetes, ADA recommends screening for DPN starting immediately at diagnosis of T2D and after 5 years duration of T1D, and annually thereafter. In both paediatric and adult subjects with diabetes, large nerve fibre dysfunction should be screened for by testing for impaired LTP, using a 10-g monofilament, and for impaired vibrotactile sense, using a 128 Hz tuning fork (24, 66, 67).

Multi-frequency vibrometry and diabetes

As mentioned above, the vibrotactile sense is often used to study large nerve fibre function of peripheral nerves among subjects with both T1D and T2D. Such examinations are mainly conducted using a 128 Hz tuning forks, but also a biothesiometer can be useful. Testing for impaired vibrotactile sense at multiple frequencies increases the likelihood of finding impaired sense since the vibrotactile sense depends on the function of Meissner's corpuscles, responding to mainly low frequency vibrations (50), and Pacinian corpuscles, responding mainly to high frequencies, optimally around 150-200 Hz (49, 51).

The multi-frequency approach to study large nerve fibre dysfunction led to the invention and development of a multi-frequency vibrometer at the Department of Hand Surgery at Skåne University Hospital in Malmö. The multi-frequency vibrometer, determining vibration perception thresholds (VPTs) in a standardized examination, was first developed to study hand-arm vibration syndrome (HAVS) among vibration exposed workers and the vibrotactile sense of subjects with compression neuropathies (68-70). Later, the use of the multi-frequency vibrometer was applied to subjects with T2D, showing that it could be a sensitive screening tool for large nerve fibre dysfunction in T2D (71). It has also been used on subjects with T1D, showing that impaired vibrotactile sense can be identified on both hands and feet (72, 73)

Pathogenesis

Although DPN is often referred to as a microvascular complication to diabetes, it is well established today that the pathogenesis of DPN is in fact multifactorial (17, 74, 75). However, the pathogenesis is not fully understood, and several pathways contribute to development of DPN.

Hyperglycaemia is the most important alteration in the homeostasis among subjects with T1D and T2D contributing to DPN development, but dyslipidaemia and insulin resistance also play important roles, where the latter two are probably more important in T2D than in T1D. The pathways to be described together contribute to oxidative stress, inflammation, mitochondrial dysfunction and altered gene expression, leading to nerve dysfunction and cell death (17).

Polyol pathway

The polyol pathway describes the process, where sorbitol and fructose are accumulated in the neuron and the peripheral nerve in response to hyperglycaemia. The accumulation of sorbitol is driven by the enzyme aldose reductase, which in the presence of hyperglycaemia creates sorbitol from excess glucose (17). This in turn leads to an osmotic stress to the neuron with subsequent loss of, for example, myo-inositol (17, 75, 76). The loss of myoinositol directly impairs nerve conduction since

myoinositol is needed for proper function of the Na/K ATPase (17, 75, 76). Furthermore, the activity of aldose reductase also increases the level of reactive oxygen species (ROSs) and this subsequently leads to oxidative stress to the cells (17, 75).

PARP pathway

Poly(ADP-ribose) polymerase (PARP) is an enzyme, with the ability to repair deoxyribonucleic acid (DNA), that can both cause and be activated by oxidative stress (75, 77). In short, activation of PARP leads to energy failure, transcriptional changes and altered gene expression in the cells (77).

AGE pathway

In a state of hyperglycaemia, the degradation of glucose will, in a non-enzymatic reaction with proteins, form advanced glycation end-products (AGEs). AGEs can cause damage to cells by altering protein functions in several ways and by activating nuclear factor kappa-B (NF-κB) through binding of AGE to the AGE receptor (RAGE) (17, 75, 76). ROS production is another consequence of AGEs binding to RAGE (75). Furthermore, AGEs can alter the extracellular matrix leading to impaired neurite outgrowth (78). Moreover, fructose, the end-product of the polyol pathway, can be converted to 3-deoxyglucosone, a precursor of AGEs, and thereby the polyol pathway interacts and contributes to the AGE pathway (76).

PKC and Hexosamine pathways

In the presence of excess glucose, the glycolysis is increased, which may have several effects on the cell. An increased glycolysis may result in accumulation of diacylglycerol (DAG), which in turn can activate protein kinase C (PKC) (17). Activated PKC can impair the function of Na/K-ATPase, with the consequence of dysfunctional nerve conduction. Furthermore, activated PKC has been shown to contribute to vasoconstriction, hypoxia and neuronal damage through altered gene expression of vascular endothelial growth factor (VEGF) and transforming growth factor-beta (TGF-β) (17, 75, 76).

Increased glycolysis will also lead to the accumulation of fructose-6-phosphate, which activates the hexosamine pathway. The hexosamine pathway involves conversion of fructose-6-phosphate and the last metabolite of the pathway is uridine 5-diphosphate-N-acetylglucosamine (GlcNac) (17, 76). GlcNac has the ability to modify various proteins, including the transcription factor Sp1, and hence has the ability to alter gene expressions and protein functions (17, 75, 76, 79).

Mitochondrial dysfunction

Glucose undergoes glycolysis and fatty acids undergo beta-oxidation prior to entering the citric acid cycle. When catabolising glucose and lipids, the citric acid cycle generates the electron donors $FADH_2$ and NAD_2. It has long been thought that

in situations with excess glucose and lipids the above-mentioned process leads to a high concentration of electron donors, i.e. a high proton gradient across the inner membrane of the mitochondrion. This may subsequently result in an electron leakage and accumulation of ROSs, due to an impaired oxidative phosphorylation. However, contradictory data exist (17, 75, 80). Normally, the level of ROSs is counterbalanced by antioxidants in the cells (17).

Oxidative stress and inflammation

Inflammation may be triggered in the cell by oxidative stress, leading to activation of several pathways, like PKC, NF-κB and cascades from mitogen-activated protein kinase (MAPK) and jun N-terminal kinase (JNK), resulting in upregulation of cytokines, such as interleukins and tumour necrosis factor-α, and chemokines (17).

Microangiopathy

As explained, although DPN is denoted a microvascular complication to diabetes, the pathogenesis depends on numerous pathways not primarily involving vascular supply to the nerves. Nevertheless, evidence showing that microangiopathy contributes to development of DPN has been reviewed and suggests for example that impaired blood flow through the microcirculation to nerves may lead to nerve damage, with a subsequent impaired regulation of the microcirculation and consequently exacerbated nerve dysfunction (81, 82). Furthermore, studies on animal models of diabetes have demonstrated improved nerve function after VEGF gene transfer, enhancing angiogenesis, suggesting a role of a blood circulation disorder in DPN development (83).

Schwann cells and DPN

Today, the relation between dysfunction of Schwann cells and DPN development is of increased interest. Schwann cells are known to cover >99% of the surface of myelinated axons, which makes the axons protected, but also reliant on the Schwann cells for energy supply since axonal connections to the extracellular room are very limited (84). The relationship between Schwann cells and axons of peripheral nerves has been reviewed by Gonçalves et al. 2017, concluding that many of the metabolic pathways discussed above, for example polyol and AGE pathways, are not only disrupting the homeostasis in the neuron itself, but also in surrounding Schwann cells (85). Goncalves et al. 2017 conclude that hyperglycaemia and dyslipidaemia contribute to Schwann cell stress, and endothelial dysfunction in surrounding microvessels, leading to neurodegeneration and impaired nerve conduction due to demyelination and loss of trophic and metabolic support, respectively (85). Thus, Schwann cells may be a key player in DPN development and an important target for future research.

Treatment of DPN

Specific disease modifying treatments for DPN in T1D and T2D have not seen the market in western countries, although several efforts have been made to develop such drugs (2). For example, the use of alpha-lipoic acid has shown promising results in some studies, but limited effect in others (2). Furthermore, aldose reductase inhibitors, interacting with the polyol pathway, have shown mild improvements of DPN in some studies, and is marketed under the name Epalrestat in for example Japan (2). However, it has failed to gain approval in, for example, the USA and many other countries.

Today the recommended treatment for DPN is focused on, according to ADA, optimizing metabolic control, i.e. glucose management, and treating pain associated with DPN (24). However, the effect of optimized glucose control on DPN is not the same for subjects with T1D and T2D (2). A comprehensive Cochrane review was carried out by Fullerton et al. in 2010, concluding that a tight glucose control was beneficial in order to delay onset of, and slow progression of, DPN, especially in early stages of the disease among subjects with T1D (86). Similar conclusions have been drawn by Martin et al. 2014 from the Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) study (19). However, in subjects with T2D an intensified glucose control has not been shown to reduce DPN (87-89). On the other hand, a small opposing study by Ohkubo et al. 1995 suggested that intensive insulin treatment, instead of conventional insulin treatment, significantly improved NCS parameters after a period of six years in subjects with T2D (90). Although there are data supporting improved nerve function by a tight glucose control among subjects with both T1D and T2D, the risk of hypoglycaemic events must be rigorously considered in order to minimize adverse events.

A brief introduction to proteomics – and other omics

In every cell of the body, there is a nucleus containing chromosomes, which are very long strains of double-stranded DNA containing the genome, i.e. the genetic code. DNA strands are a sequence of genes made up of monomers, i.e. nucleotides. The nucleotides in DNA are guanine, cytosine, adenine and thymine. Due to the molecular nature of the nucleotides, guanine can only bind to cytosine and adenine can only bind to thymine, and together they form double-stranded helices together with supporting molecules. The genetic code in a DNA string can be transcribed, i.e. read, by the formation of a single stranded ribonucleic acid (RNA) string. The RNA string is also made up of the nucleotides guanine, cytosine, and adenine, but instead of thymine the fourth nucleotide in RNA is uracil.

Forming an RNA string can be seen as copying the genetic code of a DNA string in order to translate the code into a protein, hence the process is called translation of a protein. All proteins are formed of organic compounds called amino acids. There are only 20 amino acids in total, but combining them in different orders and lengths makes it possible to create tens of thousands of proteins in the human body. Each amino acid is coded by a sequence of three nucleotides, i.e. a codon.

The technical advances in biomedical research and sciences in recent decades have vastly increased the speed and magnitude of new discoveries (91). The ability to study the whole genome has generated the field of genomics, while the field of transcriptomics presents the gene expressions of a specific cell or tissue. However, today not only the genome and transcriptome can be studied, but the entire proteome of a cell or tissue can be determined. This enables not only a description of what proteins are expressed in the cell or tissue, but also the possibility to quantify the protein expressions (91). While the genome is constant, the transcriptome and proteome are highly dynamic and change over time (91).

Heat shock proteins

For a cell to maintain protein homeostasis, chaperones are needed to properly fold new proteins to their three-dimensional structure, and to refold denatured proteins. The group of chaperones includes many HSPs. Originally the HSPs were named due to them being expressed when cells where exposed to heat-shock, but since proteins with chaperone activity exist without heat-shock induction the broader term chaperones is used instead; HSPs being a subgroup of chaperones (92). HSPs are divided into subgroups mainly depending on their mass size (93). Aside from the HSPs with confirmed chaperon activity, like HSP90, HSP70 and HSP27, there are a range of putative heat shock proteins with suspected chaperone activity.

The most studied and described HSPs regarding DPN are HSP90 and HSP70 (2, 17, 75, 94). Besides maintaining protein folding, HSP90 and HSP70 regulates the heat shock response (HSR) through its interaction with heat shock factor 1 (HSF1); HSP90 and HSP70 suppress the transcriptional activity of HSF1 by binding to it in a complex (94-96). The complex can be dissolved when a cell is subjected to numerous kinds of stress, and HSF1 can then enter the nucleus and initiate transcription of antioxidant proteins and chaperones of which one is HSP70 (94, 97, 98).

HSP27 has, in vitro, been proposed to play a role in neurite outgrowth interacting with the cytoskeleton (99), and has been associated with neuron survival following traumatic injury to peripheral nerves (100). Furthermore, in studies on T1D rats HSP27 is upregulated in the DRG following 4 months of diabetes, with concurrent decrease in nerve conduction (101). Moreover, an increased expression of HSP27

has been shown to be present in uninjured sciatic nerves among diabetic Goto-Kakizaki rats (resembling type 2 diabetes) compared to healthy rats (102). In addition, HSP27 was upregulated following nerve injury and subsequent repair, but an increase in HSP27 was not associated with alterations in axonal outgrowth (102).

In conclusion, HSPs are known guardians of the protein homeostasis in the cell and are interesting subjects to study further in DPN.

Aims

The overall aim for this thesis was to study DPN from new perspectives. This was done by:

1. Using MFV to study signs of impaired vibrotactile sense, mirroring DPN, in a cohort of paediatric subjects with T1D.
2. Describing the full proteome of PIN from subjects with type 1 and type 2 diabetes, and healthy controls.

The specific aims for the papers included in the thesis were as follows.

Paper I: The aim of this study was to evaluate VPTs, obtained with a Vibrosense Meter, and LTP, using Semmes-Weinstein's monofilament, from children and adolescents with T1D in order to investigate whether impaired sense, reflecting DPN, could be identified.

Paper II: The aim was to investigate potential associations between impaired vibrotactile sense in children and adolescents with T1D and the presence of IA-2 and/or GAD65 autoantibodies.

Paper III: The aim of this study was to re-examine children and adolescents with T1D with the Vibrosense Meter in order to study the variability of the vibrotactile sense.

Paper IV: The aim was to present quantitative mass spectrometry data from human peripheral nerves (PIN) obtained from elderly male subjects with and without T2D.

Paper V: This paper aimed to present and investigate the quantitative presentation of HSPs in PIN from subjects with T1D, T2D and healthy controls, and to investigate whether the protein expression of HSPs could be associated with type of diabetes and/or electrophysiology and morphometry data.

Methods

Study populations

Papers I-III – the vibrometry studies

The 72 subjects comprising the study population in Papers I-III are paediatric T1D subjects recruited from the paediatric outpatient clinics at Skåne University Hospital in Malmö and Lund, and from Helsingborg Hospital. They were recruited between April 2015 and June 2016. In order to be part of the study, subjects needed to be aged 8-18 years and be diagnosed with T1D. Subjects with concomitant diseases known to, or suspected to, cause impaired nerve functions were excluded.

A normal material for VPTs, previously collected from healthy children and adolescents (103), was used to compare VPTs obtained from T1D subjects. The normal material was collected from 283 healthy children and adolescents (girls: n = 146), aged 8 to 20 years, invited from three different schools (103). The normal material is divided into groups based on gender and subgroups depending on age category; i.e. 8-10 years, 11-15 years and 16-20 years (103)

Written informed consents were obtained from all participants, as well as their legal guardians. The local ethics committee at Lund University approved the vibrometry studies (386/2007).

Papers IV and V – the PIN studies

In Paper IV, twelve (T2D: n=9; healthy controls: n=3) out of totally 15 subjects were recruited from a study on older male subjects with and without T2D originally participating in a prospective health screening study meticulously followed-up for several years in Malmö, Sweden (58). The remaining three healthy controls were recruited from a study on otherwise healthy subjects, and subjects with diabetes, with carpal tunnel syndrome (CTS) undergoing carpal tunnel release with concurrent PIN biopsy (55).

In Paper V, a total of 56 subjects were included, of whom 15 were also part of Paper IV. Out of the 56 subjects, recruited from the two abovementioned studies (55, 58),

nine were diagnosed with T1D, 24 were diagnosed with T2D and 23 were healthy controls.

Ethical permits were granted for the PIN studies by the local ethics committee at Lund University (LU508-03 and LU504-03), and written informed consents were obtained from all subjects.

Screening tools

Multi-frequency vibrometry

In Papers I and III, the vibrotactile sense of children and adolescents with T1D was examined using a Vibrosense meter (Vibrosense Dynamics, Malmö, Sweden) (104, 105). The MFV device runs a standardised program examining the vibrotactile sense for seven frequencies (8, 16, 32, 64, 125, 250 and 500 Hz) on two sites of the right hand and five frequencies (8, 16, 32, 64 and 125 Hz) on two sites of the right foot. On the right hand, the pulps of index and little fingers are examined, and on the right foot, the examination is carried out at metatarsal heads one (MTH1) and five (MTH5). The examination provides a vibrogram, seen in Figure 1, depicting the vibrotactile perception, but also numerical values of the VPTs.

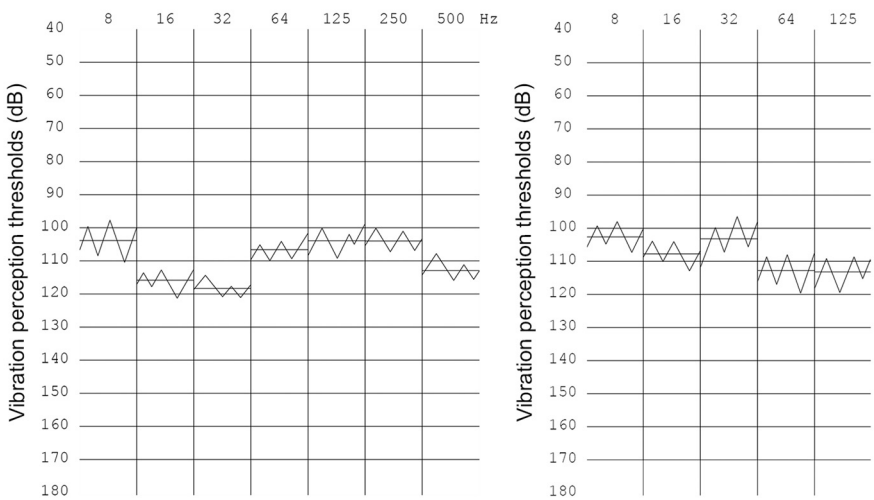

Figure 1. Normal vibrograms from hand and foot.
The vibrograms above show VPTs obtained from one subject on the hand (vibrogram to the left) and from another subject on the foot (vibrogram to the right). VPTs are obtained at seven frequencies (8, 16, 32, 64, 125, 250 and 500 Hz) on the hand and on five frequencies (8, 16, 32, 64 and 125 Hz) on the foot. The frequencies examined are shown at the top of the x-axis. On the y-axis the VPT is shown in an inverted manner with higher VPTs lower in the vibrogram. VPTs are measured in decibel (dB).

The numerical values of VPTs from the vibrograms are comparable to other subjects, as described in Figure 2, but also to our previously collected normal material (103), which can be used to calculate z-scores of VPTs based on age and gender.

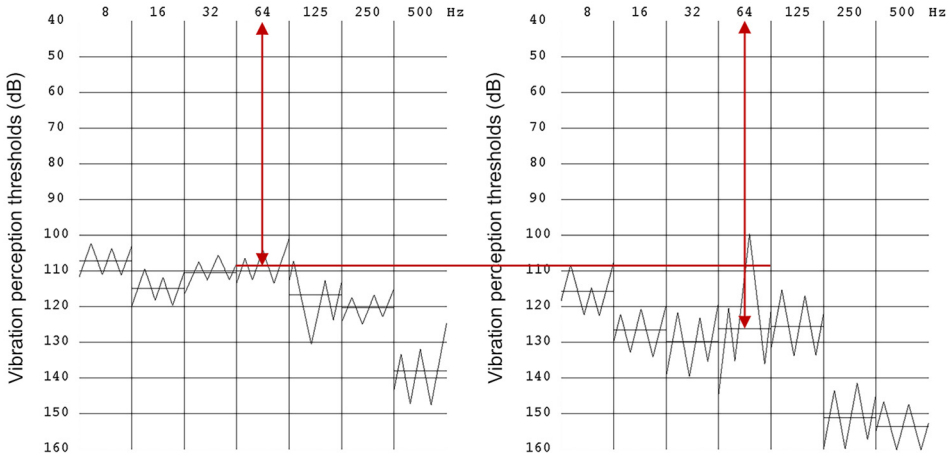

Figure 2. Examples of vibrograms depicting normal and impaired vibrotactile sense.
The vibrograms above are from two different subjects. Examinations were performed on the right hand in both subjects and subjects are of equal age and of the same gender. The vibrogram to the left depicts VPTs within the normal range for that age group on all frequencies tested. The vibrogram to the right shows elevated VPTs on all frequencies, which is highlighted at 64 Hz with the read arrows. Please note that the scale is inverted, with the highest VPTs in the lower parts of the vibrogram. Picture adapted from a text by the author of this thesis in the journal Sticket 2018 (106) (the author holds copyright).

A thorough description of the MFV method can be found in Paper I (104), and a brief description will follow. The test person sits in a secluded room wearing headphones to maintain a calm environment. The examined hand or foot lies rested on a surface with a vibrating probe eliciting vibrations at the aforementioned frequencies. When the test person perceives the vibrations, they press the button on a remote control, and when the vibration ceases the button on the remote control is released. If the test person does not feel the vibration, the vibrating probe will start to vibrate with a greater intensity, maintaining its frequency. Thus, a threshold is generated once the vibration is perceived. The experimental set-up is presented in Figure 3.

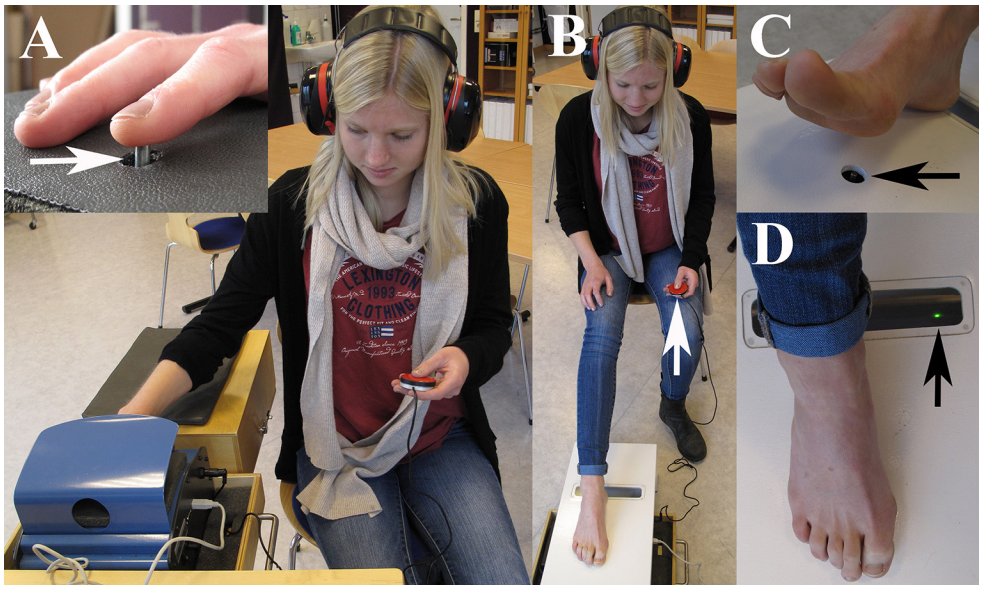

Figure 3. Experimental set-up of MFV examinations.
The person being examined sits in a secluded room wearing headphones to reduce external stimuli. The examined hand is held in a resting position with the finger being examined (index or little finger) put on the vibrating probe shown in (A). When the test person perceives vibrations on the examined hand or foot, he/she presses the button on the remote control (B). When the vibration can no longer be perceived the test person releases the button on the remote control. The probe for examination of the foot (at metatarsals head one and five) is shown in (C). The green indicator shown in (D) shows that the pressure to the vibrating probe is appropriate, i.e. not too hard or too loose. Picture adapted from Dahlin et al. 2015 (open-access) (103).

In order to include a vibrogram for statistical analyses, the tested site needed to have at least three visibly correct frequencies, defined in Ising et al. 2018 (104) meaning that the VPTs of those frequencies had at least 5 endpoints in a structured pattern without outlying endpoints, indicating, for example, lack of concentration. Z-scores of the VPTs were calculated in reference to previously collected normative data from 269 healthy children and adolescents (103). All vibrograms were manually scrutinized to exclude curves with signs of lack of concentration or failure to comply with the examination at a certain frequency (Figure 4).

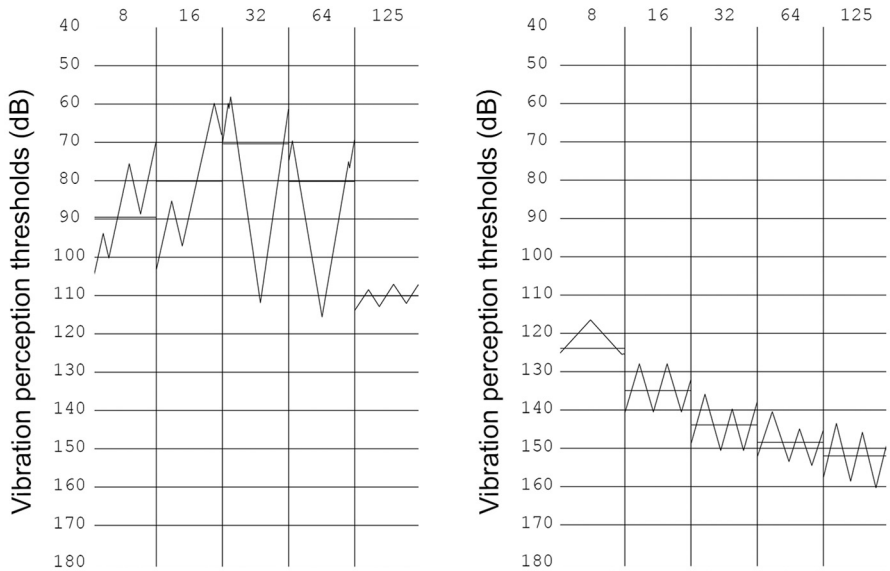

Figure 4. Examples of an inadequate vibrogram and an incomplete frequency.
The vibrogram to the left clearly shows that the test person has failed to comply with the examination. The pattern of the VPTs are unstructured in frequencies 8, 16, 32 and 64 Hz, but looks appropriate at 125 Hz. The vibrogram to the left is an example of an examination that is considered inadequate, and hence excluded prior to statistical analyses. In the vibrogram to the right, the VPT at 8 Hz only has three endpoints and has therefore been excluded prior to statistical analyses.

Monofilaments

Screening for DPN through the perception to light touch was carried out in Papers I and III on the same sites as vibrotactile testing using a full 20 pieces Semmes-Weinstein's monofilament collection (Touch-Test®, North Coast Medical Inc., Morgan Hill, Ca, USA). The monofilaments range from 0.008-g to 300-g, including the 10-g (i.e. 5.07) monofilament, which is the sole monofilament recommended for screening of DPN in both children, adolescents and adults (59, 66).

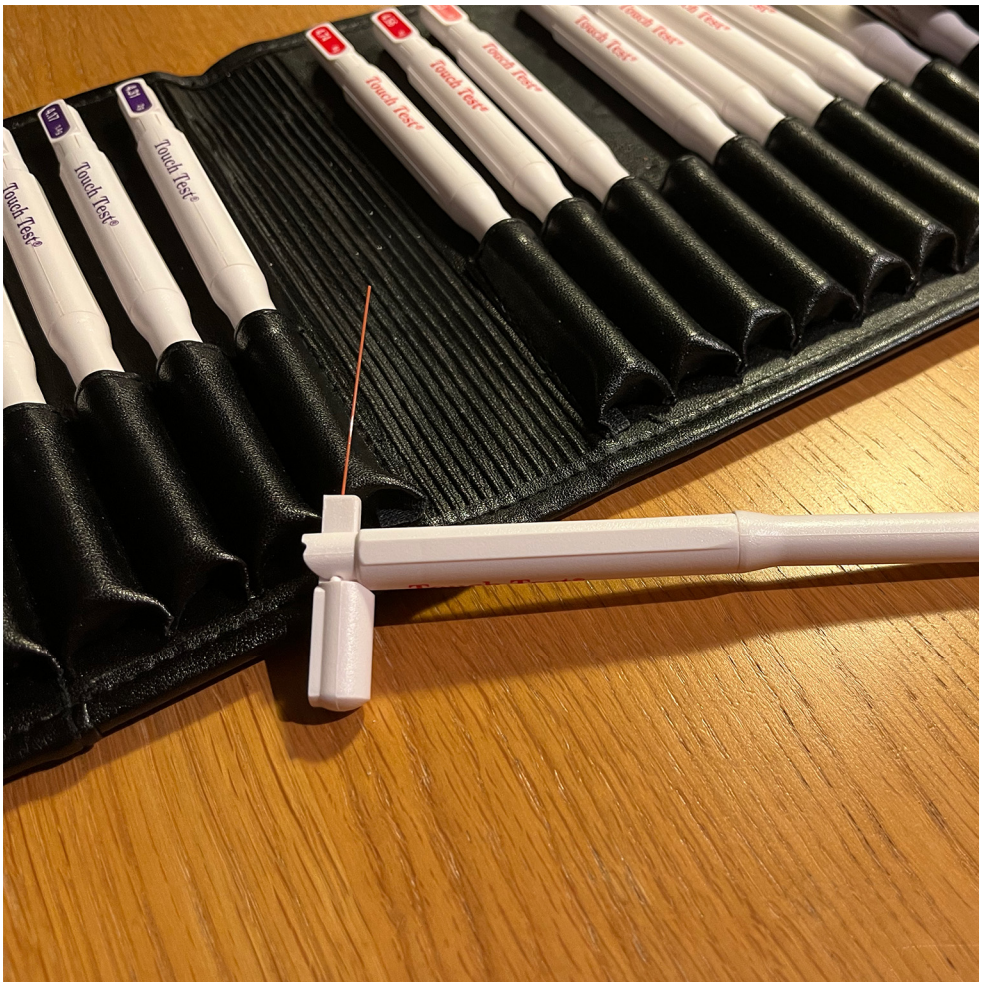

Figure 5.
A 10-g (i.e. 5.07) monofilament is shown (part of the Touch-Test® manufactured by North Coast Medical Inc., Morgan Hill, Ca, USA). Author's own picture.

Autoantibodies in T1D

In Paper II the presence of autoantibodies to T1D, from clinical onset of disease, among the study population of Paper I were related to the subjects' vibrotactile sense. I chose to study the relationship between GAD65 and/or IA-2 autoantibodies and impaired vibrotactile sense.

Autoantibody data was collected from two different sources and analyses were carried out at different laboratories. Therefore, the titres of GAD65 and IA-2 were

not comparable to each other and only presence or absence of the autoantibodies was used in Paper II. The presence of GAD65 and IA-2 autoantibodies was studied in-house using a radio binding assay as described by Delli et al. 2012 (107) and at Klinisk Kemi, Medicinsk Service at Skåne University Hospital, Malmö, Sweden, using either a radio binding assay or enzyme-linked immunosorbent assay (ELISA) (108, 109).

Posterior interosseous nerve

PINs from healthy living human donors, and from living human donors with T1D or T2D, were used in Papers IV and V. PINs were harvested under local or regional anaesthesia, and with a bloodless operative field as described by Thomsen et al. 2009a (54). The full PIN is around three to four cm of length and was divided into four parts for different analyses. One of those pieces, around one cm of length, was used in Papers IV and V. This one cm long piece was cut into two equal parts. Thus, each specimen used in Papers IV and V were 5 mm long, weighing approximately 5.8 mg each.

Proteomics - mass spectrometry preparation and analysis

The full technical description of sample preparation, liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis and subsequent data analysis can be found in Paper IV (110). However, a brief summary follows.

Fresh frozen full nerve tissue pieces were used for LC-MS/MS analysis. Tissues were suspended in a buffer with ammonium bicarbonate and urea in order to unfold proteins and maintain pH. Samples were then homogenized and thereafter reduced using tris-2-carboxyethyl phosphine, in order to break disulphide bonds, and alkylated with iodoacetamide, leading to maintained unfolded proteins. Extracted proteins were then digested, using trypsin, into peptide sequences and thereafter desalted. Desalted samples were dried and resuspended in acetonitrile and formic acid.

Peptides were separated using a high-performance liquid chromatography (i.e. HPLC) system. Both data-dependent acquisitions (i.e. DDA) and data independent acquisitions (i.e. DIA) were used for quantitative proteomic analyses.

Electrophysiology

In Papers IV and V, electrophysiology data, i.e. NCS data, from the upper and lower extremities were used. The set-up for electrophysiology testing is shown in Figure 6. Sural nerve sensory conduction velocity (sSCV), sural nerve amplitude (sSAMP) and peroneal nerve motor conduction velocity (pMCV) were obtained.

Figure 6.
Set-up for electrophysiology testing. Picture adapted from the doctoral thesis by Malin Zimmerman, and is reused by due permission from the author (111).

Furthermore, since PINs are situated in the upper extremity, in the distal forearm, electrophysiology data from the ulnar nerve was obtained as well. At wrist level, ulnar sensory amplitude (uSAMP), ulnar sensory conduction velocity (uSCV) and ulnar motor amplitude (uAMP wrist) were obtained. Moreover, ulnar motor conduction velocity (uMCV) and ulnar distal motor latency (uDML) were examined. An example of a nerve conduction study performed on the upper extremity is shown in Figure 7.

Figure 7.
A nerve conduction study being performed on the upper extremity. Picture adapted from the doctoral thesis by Malin Zimmerman, and is reused by due permission from the author (111).

Statistical analyses

Paper I

All statistical analyses were carried out using IBM SPSS Statistics version 23 for Mac (IBM Corp, Amonk, NY, USA). Due to non-normally distributed data and small sample sizes, I present median and quartiles for all characteristics and VPTs. Differences in characteristics and VPTs were calculated using Mann-Whitney U-tests. VPTs were presented as z-scores compared to previously collected normative data (103). A Bonferroni correction was used due to multiple comparisons regarding VPTs. To test association between characteristics and elevated VPTs, i.e. a pathological examination due to criteria previously stated, Chi2 with Fisher's exact tests were used. On all statistical tests, p-values, or corrected p-values when Bonferroni was used, < 0.05 were considered statistically significant.

Paper II

In Paper II potential associations between the presence of autoantibodies to GAD65 and/or IA-2 and impaired vibrotactile sense in the subjects from Paper I were analysed. Associations were calculated using Chi2 with Fisher's exact test. All analyses were performed in IBM SPSS Statistics version 25 for Mac (IBM Corp, Amonk, NY, USA).

Paper III

In this follow-up study on vibrotactile sense, I presented general characteristics as medians and quartiles, and comparisons between baseline and follow-up were carried out using Mann Whitney U-tests. Differences in VPTs, on all frequencies, between baseline and follow-up were tested using Wilcoxon signed rank tests with Bonferroni corrections applied on significant values. Significance was considered as $p < 0.05$ after correction. Furthermore, a mean of all VPTs at a specific site was calculated in order to compare an entire site from baseline with follow-up. Mean VPT z-scores from baseline were compared to follow-up using Wilcoxon signed rank test and Spearman's correlations were used to test for correlations between mean VPT z-scores and HbA1c. All analyses were performed in IBM SPSS Statistics version 27 for Mac (IBM Corp, Amonk, NY, USA).

Paper IV

Characteristics were presented as medians and quartiles, and differences in characteristics between subjects with T2D and healthy controls were tested using Mann Whitney U-tests, with $p < 0.05$ considered statistically significant. Electrophysiology data was also presented using medians and quartiles, and a linear regression model was used on electrophysiology data that differed between subjects with T2D and healthy controls in order to control for confounders (i.e. age and BMI). All analyses regarding characteristics and electrophysiology were performed using IBM SPSS Statistics version 26 for Mac (IBM Corp, Amonk, NY, USA).

In close collaboration with a statistician, protein intensities obtained from PIN in subjects with T2D and healthy controls were analysed. Firstly, intensities were log2 transformed and normalized. To look for differentially expressed proteins between groups a linear regression model was primarily used, but since no statistically significant differences were seen, the proteins were further analysed using the 500 proteins with the highest variance across all samples. On these proteins, an unsupervised hierarchical clustering with complete linkage was used to look for clusters of similar proteins. All analyses of protein intensities were carried out using R version 3.5.1 (R core team, R Foundation for Statistical Computing, Vienna, Austria)

Paper V

Medians and quartiles were used to present characteristics, and differences between groups (T1D, T2D and healthy controls) were analysed using Kruskal-Wallis test with post-hoc Mann-Whitney U-tests on significant values. Mann-Whitney U-tests were used when comparing characteristics between two groups. $P < 0.05$ was considered statistically significant. IBM SPSS Statistics version 27 for Mac (IBM Corp, Amonk, NY, USA) was used for analyses of characteristics.

Protein analyses were performed using R version 4.1 (R core team, R Foundation for Statistical Computing, Vienna, Austria). Linear regression was used to examine if any proteins were differentially expressed between subjects with T1D, T2D and healthy controls. An unadjusted linear regression model, as well as a regression model adjusting for age, gender and BMI was used. Correlations between protein intensities and nerve conduction data from the ulnar nerve was analysed using Spearman's correlations. To adjust for multiple comparisons all p-values were corrected using Benjamini and Hochberg (false discovery rate) corrections.

Results

Below, the results of each paper are briefly summarized. The reader is referred to the full text of the specific papers for further information.

Paper I

As intended, vibrotactile sense was tested using MFV and sensation to light touch was tested, using Semmes-Weinstein's monofilaments. Examinations were performed on 72 (boys = 39) children and adolescents with T1D, aged in median [quartiles] 12.8 [11.5-15.0] years. The median disease duration was 5.3 [2.9-8.6] years and median HbA1c among subjects was 57 [50-62] mmol/mol.

A total of 13 out of 72 subjects showed signs of impaired vibrotactile sense on at least one site on the foot, but none of the subjects showed signs of impaired sense to light touch. Z-scores of VPTs, compared to normative values previously collected by Dahlin et al. 2015 (103), showed an increased proportion of pathological VPTs, i.e. z-score >1.96, than expected. In Figure 8 the number of subjects with a z-score of >1.96 at every frequency and site is shown. Elevated VPTs were especially apparent at low frequencies on the foot. Furthermore, pathological VPTs were seen more frequently among subjects treated with insulin using MDI compared to subjects treated with CSII. No such differences were seen regarding age, disease duration, HbA1c or gender.

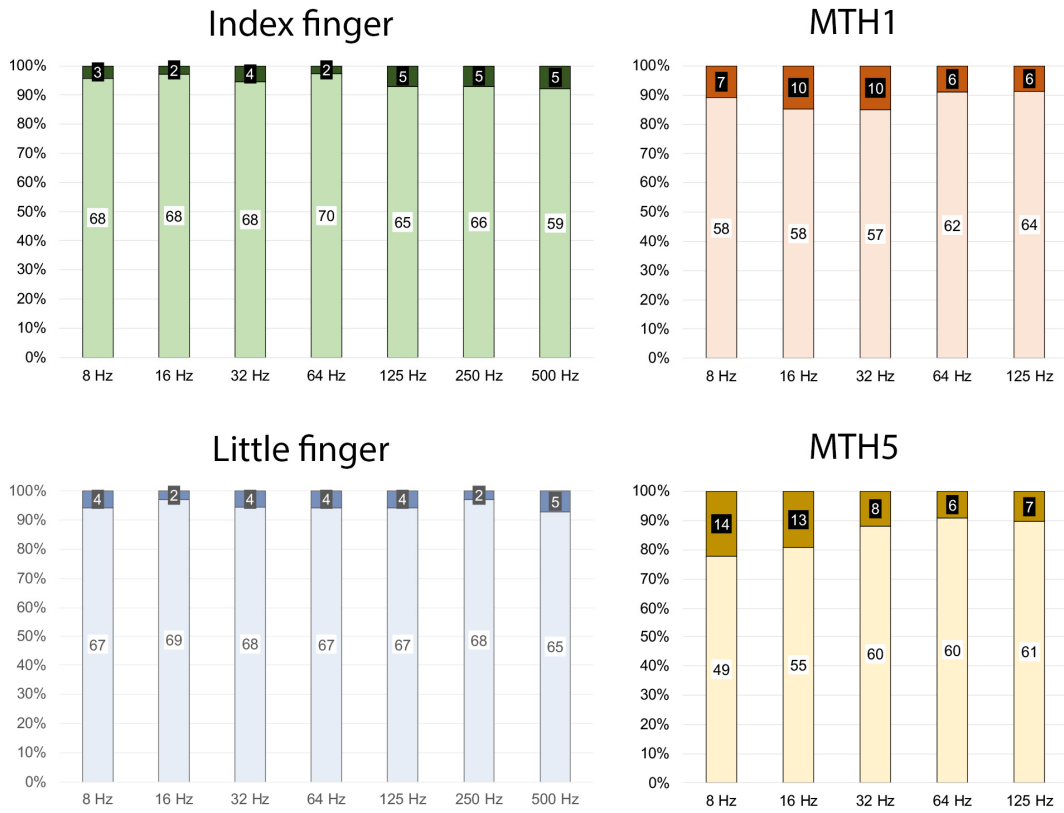

Figure 8. Distribution of elevated VPTs at every frequency and site.
The number of subjects presented with an elevated VPT (i.e. >1.96 SD) is shown for every site (index finger, little finger, MTH1 and MTH5) and frequency (8,16, 32, 64, 125, 250 and 500 Hz on the hand and 8, 16, 32, 64 and 125 Hz on the foot). The figure is adapted (recoloured) from Ising et al. 2018 (104) (open access).

Paper II

After Paper I was finished, I was provided with data on the presence of autoantibodies to GAD65 and IA-2 in 70 out of the 72 children and adolescents participating in the study from Paper I, including 12 out of the 13 subjects showing signs of impaired vibrotactile sense. An analysis was carried out testing a possible association between the presence of autoantibody(-ies), from the time of T1D diagnosis, and impaired vibrotactile sense. Using Fisher's exact test, I did not see any association between impaired vibrotactile sense and the presence of autoantibodies to GAD65, IA-2 or both.

Paper III

Since DPN development in T1D has been shown to be associated with disease duration and HbA1c, I aimed to follow up the subjects from the study in Paper I after approximately two and a half years with a new MFV examination to see what happens with the vibrotactile sense over time.

A total of 37 subjects, out of originally 72 in Paper I, accepted follow-up in this study. The median [quartiles] follow-up time was 30 [25-32] months. Baseline HbA1c of the participants of the follow-up study was slightly lower compared to non-attenders (54 [50-58] vs 59 [51-67] mmol/mol, $p = 0.024$). Comparing VPTs at follow-up with baseline showed that VPTs were not higher (i.e. indicating deterioration of vibrotactile sense) at any site or frequency, but rather lower at low frequencies on the foot at follow-up compared to baseline. At the same time, no subjects showed impaired sense to light touch at baseline, but four out of 37 subjects presented with impaired sense to light touch at follow-up. No associations were seen between VPTs and HbA1c at follow-up.

Paper IV

In Paper IV, I aimed to approach DPN from a new perspective by describing the full proteome of PIN in subjects with T2D, showing early signs of DPN, and healthy controls, and to examine for any differences in protein expressions between groups.

A total of 15 subjects (T2D: $n = 9$; healthy controls: $n = 6$) participated in the study. Subjects who originally participated in a large prospective health screening study, or in a study on subjects with CTS undergoing carpal tunnel release, were included and PINs were harvested (55, 58).

Using LC-MS/MS, I quantified a median of 2543 proteins, with a false discovery rate of 1%, in PIN, and I could identify a total of 2617 different proteins across all samples. A linear regression model did not determine any significant differences regarding differentially expressed proteins between groups. Proteins of importance to DPN development were identified. For example, proteins connected to extracellular matrix and the cytoskeleton, like collagens and tubulins, were prominent. Furthermore, proteins associated with lipid metabolism (for example apolipoproteins), myelin formation (myelin basic protein and myelin protein zero) and protein homeostasis (HSPs) were also detected.

Paper V

The aim of Paper V was to describe the quantitative expression of HSPs among subjects with T1D, T2D and healthy controls, and to study potential associations of protein expression to type of diabetes as well as electrophysiology from the upper extremity (i.e. ulnar nerve) and morphometry of PIN.

A total of 56 subjects participated in the study [T1D: n = 9 (female = 6); T2D: n = 24 (female = 7); controls: n = 23 (female = 12)]. Study participants were previously part of two different cohorts (55, 58). Median [quartiles] age was 46 [39-54] and 69 [61-74] years for T1D and T2D respectively, and 57 [46-68] for controls, and differed significantly between groups (Kruskal-Wallis test: $p < 0.001$). T1D subjects had significantly longer disease duration than T2D subjects (28 [13-38] vs 10 [3-15] years; Mann-Whitney U-test: $p = 0.003$) and significantly higher HbA1c (58 [55-78] vs 46 [36-57] mmol/mol; Mann-Whitney U-test: $p = 0.006$).

For the full presentation of electrophysiology and morphometry data please see Paper V. T2D subjects had significantly lower conduction velocities and amplitudes compared to controls on all electrophysiological parameters in the ulnar nerve. T1D subjects had significantly lower uMCV than controls, but all other parameters did not differ between T1D and controls in the ulnar nerve. Myelinated nerve fibre density did not differ between groups.

A total of 32 different HSPs and putative HSPs were identified and quantified in PIN (Table 1). Using linear regression, I did not see any statistically significant differences in protein expression for any of the proteins between groups when adjusting for multiple comparisons using false discovery rate. However, several proteins of the HSP90 family, as well as HSP70, approached statistical significance regarding differences in protein expression between groups. Boxplots of protein expressions, of selected proteins, between groups can be seen in Figure 9.

HSP 70 superfamily: HSPA (HSP 70) and HSPH (HSP110) families

Subgroup	Name	Gene	Uniprot
HSPA	Heat shock 70 kDa protein 1A (HSP70-1/HSP72)	HSPA1A	P0DMV8
	Heat shock 70 kDa protein 1-like	HSPA1L	P34931
	Heat shock-related 70 kDa protein 2	HSPA2	P54652
	Heat shock 70 kDa protein 4	HSPA4	P34932
	Heat shock 70 kDa protein 4L	HSPA4L	O95757
	Endoplasmic reticulum chaperone BiP	HSPA5	P11021
	Heat shock 70 kDa protein 6	HSPA6	P17066
	Putative heat shock 70 kDa protein 7	HSPA7	P48741
	Heat shock cognate 71 kDa protein	HSPA8	P11142
	Stress-70 protein, mitochondrial	HSPA9	P38646
	Heat shock 70 kDa protein 12A	HSPA12A	O43301
	Heat shock 70 kDa protein 12B	HSPA12B	Q96MM6
HSPH	Heat shock protein 105 kDa	HSPH1	Q92598

The DNAJ family (HSP40)

Subgroup	Name	Gene	Uniprot
DnaJA	DnaJ homolog subfamily A member 2	DNAJA2	O60884
DnaJB	DnaJ homolog subfamily B member 2	DNAJB2	P25686
	DnaJ homolog subfamily B member 4	DNAJB4	Q9UDY4
DnaJC	DnaJ homolog subfamily C member 11	DNAJC11	Q9NVH1

The HSPB family (small heat shock proteins)

Subgroup	Name	Gene	Uniprot
HSPB	Heat shock protein beta-1 (HSP27)	HSPB1	P04792
	Heat shock protein beta-6	HSPB6	O14558
	Heat shock protein beta-8	HSPB8	Q9UJY1

The HSP90/HSPC family

Subgroup	Name	Gene	Uniprot
HSPC	Heat shock protein HSP 90-alpha	HSPC1/HSP90AA1	P07900
	Heat shock protein HSP 90-alpha A2	HSP90AA2P	Q14568
	Putative heat shock protein HSP 90-alpha A5	HSP90AA5P	Q58FG0
	Heat shock protein HSP 90-beta	HSPC3/HSP90AB1	P08238
	Putative heat shock protein HSP 90-beta 2	HSP90AB2P	Q58FF8
	Putative heat shock protein HSP 90-beta-3	HSP90AB3P	Q58FF7
	Putative heat shock protein HSP 90-beta 4	HSP90AB4P	Q58FF6
	Endoplasmin	HSPC4/HSP90B1	P14625
	Putative endoplasmin-like protein	HSP90B2P	Q58FF3
	Heat shock protein 75 kDa, mitochondrial (TRAP1)	HSPC5/TRAP1	Q12931

Chaperonins and related genes

Subgroup	Name	Gene	Uniprot
HSPD	60 kDa heat shock protein, mitochondrial	HSPD1	P10809
HSPE	10 kDa heat shock protein, mitochondrial	HSPE1	P61604

Table 1.
Identified HSPs from PIN, including putative HSPs. Adapted from Paper V (unpublished manuscript).

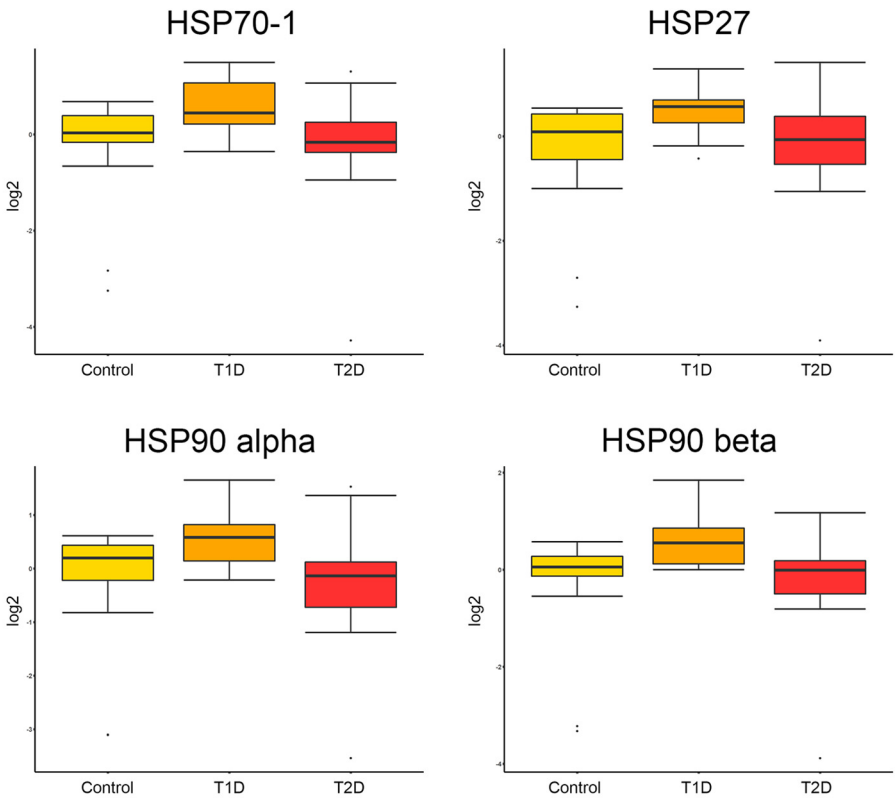

Figure 9.
Boxplots showing the protein intensities of selected HSPs. Adapted from Paper V (unpublished manuscript).

General discussion

The overall aim of this thesis, to approach DPN from new perspectives, relies on two main considerations. These are a) finding DPN at early stages in order to raise awareness of the complication for the affected young subjects, and b) approaching the pathogenesis of DPN from a new perspective in order to aid in the future search for biomarkers of DPN and potential agents for drugs targeting DPN. The two main considerations are intertwined since the lack of drugs targeting DPN has led to a need of finding DPN early to minimise the long-term consequences.

Diabetic peripheral neuropathy is common among both paediatric and adult subjects with T1D and T2D and guidelines recommend screening for this severe complication annually using a 10-g monofilament and a 128 Hz tuning fork alongside a thorough clinical examination (24, 66, 67). As will be discussed below, screening with monofilament and tuning forks are quick and easy but lack sensitivity and may be insufficient at finding early stages of DPN (112-114). Furthermore, the diagnostic accuracies of these simple screening tools have been shown to be limited, especially in paediatric subjects (61, 63, 115, 116), possibly due to lack of consensus regarding examination protocols (117). Considering the difficulties of screening for DPN with the present screening tools, I wanted to approach screening from a new perspective using MFV.

Although much is known about the pathogenesis of DPN, a lot remains to be explained. Though several attempts have been made to find disease modifying treatments of DPN, the results have been scarce. A general dilemma of studying pathophysiologic aspects of DPN is that the harvesting of appropriate nerve specimens is invasive. The most common nerve to be biopsied for DPN studies has been the sural nerve, which involves risks of residual problems (118, 119). Thus, most research is conducted in vitro or in animal models of diabetes. Although several animal models exist that reflect the environment of either T1D or T2D, they still differ significantly from human diseases (17, 120). However, taking biopsies of the presently used nerve (i.e. PIN; used in Papers IV-V) results in no risks (aside from a small chance of wound infection) of residual problems in humans according to Thomsen et al. 2009a (54).

DPN in children and adolescents with T1D

From studies using NCS (i.e. electrophysiology), the gold standard method for detecting abnormal peripheral nerve function, it has been established that DPN not only is present among children and adolescents with T1D, but is also common (13, 61, 121). Numerous studies have shown that DPN is often subclinical in young subjects with T1D, and that subclinical DPN may present early in the course of diabetes (29, 122-126). Following an increase in prevalence of T1D, the prevalence of DPN is expected to rise among young T1D subjects (127). A recent review by Rasmussen et al. 2021 estimated the prevalence of large nerve fibre dysfunction, as determined by abnormal NCSs from five different studies, to be 10-57 % among subjects with T1D aged 10 to 19 years (62). However, it is hard to ascertain the prevalence of DPN among subjects with T1D from present studies due to several factors; different nerves have been used in NCS, the age intervals used in the studies vary and different electrophysiological parameters, cut-offs and normative data have been used (62). For example, in a study by Riihima et al. 2001, paediatric subjects with T1D aged > 9 years, and with disease duration of at least 2 years, were included. The results from NCSs were compared to age- and gender-matched controls (128). Riihima et al. 2001 showed a prevalence of DPN of 10 %, based on criterion that ≥ 2 NCS parameters were above the 99th or below the 1st percentile of NCS values from controls (128). In a study by Nelson et al. 2006, on paediatric T1D subjects, DPN was defined as having NCS values below 2 standard deviations (SDs) of the mean from reference values in two different nerves. With these criteria a 57 % prevalence of DPN was shown (116). The different criteria aside, characteristics of T1D subjects in the study by Nelson et al. 2006 were similar to the characteristics in Riihima et al. 2001 regarding age, disease duration and HbA1c. However, Nelson et al. 2006 did not report on puberty state, while Riihima at el. 2001 showed increasing large nerve fibre dysfunction with increasing Tanner stage (116, 128). These studies indicate that the presence of DPN may vary extensively between cohorts, but also that different definitions of DPN makes it hard to compare cohorts, and even harder to conduct meta-analyses on the prevalence of NCS verified DPN in children and adolescents with T1D.

In Paper I, 18 % of the subjects showed signs of impaired vibrotactile sense suggesting early signs of DPN, which is within the range of DPN prevalence suggested by Rasmussen et al. 2021 (62). However, it must be stated that the definition of impaired vibrotactile sense chosen in Paper I is also somewhat arbitrary. I chose to define a pathological MFV examination as having a z-score [calculated in comparison with previously collected normative data (103)] of > 1.96 for at least three frequencies, on at least one of the sites examined (i.e. index finger, little finger, MTH1 or MTH5). A z-score of > 1.96 reflects the top 2.5% outside a two-sided 95 % confidence interval. It can be argued that the use of a two-sided confidence interval is not the most appropriate in this case. Due to the central limit

theorem, I then claim that, with 95% certainty, the true value in the population lies within 1.96 SDs from the mean. However, in terms of abnormal nerve function it is not interesting to study the subjects outside the lower limits of the confidence interval, since their nerve function is better than expected. Thus, using a one sided 95% confidence interval could arguably be more correct. In that case, the z-score cut-off should instead have been 1.64, reflecting the top 5% of a one-sided 95% confidence interval. Furthermore, setting the number of abnormal frequencies to at least three is also quite conservative. With this in mind, the prevalence of impaired vibrotactile sense in Paper I could be influenced by the cut-offs, which will potentially underestimate the number of subjects with impaired vibrotactile sense. In conclusion, the cut-offs and level of confidence could be adjusted, both up and down, in order to reduce the number of false negative or false positive examinations. For the future, my suggestion would be to conduct a study on children and adolescents with T1D using MFV and NCSs to compare MFV to the gold standard. Nevertheless, I find the use of MFV as a screening tool for DPN promising, which will be discussed further below.

Screening of DPN in children and adolescents with T1D

Screening of DPN among children and adolescents is important and should be conducted early in the course of diabetes, but the psychosocial aspects of screening for an untreatable complication must be highlighted. At best, knowledge of the complication can hopefully help to motivate patients to take care of their diabetes with greater strictness in cases of insufficient metabolic control, but at worst that knowledge may also be associated with concerns and anxiety. It has previously been shown by Hood et al. 2014, from the SEARCH for Diabetes in Youth Study, that deteriorating quality of life is associated with impaired metabolic control (129). Therefore, I believe in the multidisciplinary, i.e. team-based, approach in the care for T1D subjects focusing not only on somatic, but also mental, well-being, as is the standard of care in Sweden and many other countries. However, it has been difficult to prove the effectiveness of such strategies, but much of the concerns have been due to methodological issues and failure to meet the needs of the patients (130, 131). Although DPN is often subclinical in paediatric T1D subjects (124, 132, 133), and hence will probably not lead to foot ulcers in the foreseeable future, a tight glucose control due to intensified insulin treatment may slow the progression of DPN (19). Furthermore, it has been suggested by Ziegler et al. 2021 that nerve dysfunction among subjects with recent onset T1D may actually be reversible five years from baseline (134). It has also been shown that vibrotactile sense might improve over time in adult T1D subjects with improved metabolic control (135), which is in accordance with the results of Paper III that will be further discussed below.

As described earlier in the background section, international clinical guidelines recommend screening children and adolescents with T1D for large and small nerve

fibre dysfunction (66, 67). ISPAD recommends screening from 11 years of age and with two to five years of disease duration (67), while ADA recommends screening from 10 years of age and with 5 years of disease duration (66). Both ISPAD and ADA recommend that screening of large nerve fibre dysfunction includes examination of sensation to light touch, using a 10-g monofilament, and vibrotactile sense (66, 67). ADA has specified that screening of vibrotactile sense should include the use of a 128 Hz tuning fork (59, 66), while ISPAD has not specified what tools they recommend (67). Using a 10-g monofilament to screen for impaired sense to light touch among paediatric subjects with T1D, a group where DPN is mostly subclinical (122, 123), is in my opinion controversial, since the 10-g monofilament has mainly been established to be useful at finding severe cases of DPN (113). Based on my thesis, as well as the literature I have reviewed writing it, my recommendation would be to start screening for DPN early among subjects with T1D. I recommend starting screening for DPN as soon as the subjects can co-operate with the examinations, but latest at the ages recommended by ADA and ISPAD. In my studies, subjects were included from the age of 8 years, and the great majority of the youngest subjects had no problems co-operating. Furthermore, I recommend that screening for large nerve fibre dysfunction includes screening of both low and high vibration frequencies, using multi-frequency vibrometry as a suggestion, and that screening of impaired LTP is conducted using a large monofilament testing kit, and not only the 10-g monofilament.

Screening using monofilaments

In Papers I and III, I used Semmes-Weinstein's monofilaments, i.e. the Touch Test®, and used normative values suggested by the manufacturer (136, 137). The Touch Test® includes 20 different monofilaments from size 1.65 (0.008-g) to 6.65 (300 g). Impaired LTP to the 3.61 (0.4-g) monofilament is described as diminished perception to light touch in hand, while impaired LTP to the 3.84 (0.6-g) monofilament is the corresponding monofilament for the plantar side of the foot. Impaired LTP, resulting in loss of protective sensation, is defined as the inability to perceive the 4.56 (4-g) and the 5.07 (10-g) monofilaments on the hand and plantar foot, respectively. All suggested discrimination levels by the manufacturer are based on normative values from adults, making it difficult to adapt them to my studies of children and adolescents. However, in Papers I and III, I used the full 20-pieces monofilament set, which should be much more accurate in finding early signs of LTP than the recommended 10-g monofilament, which is mainly testing loss of protective sensation (24, 113).

As presented earlier, none of the 72 paediatric participants in Paper I had impaired LTP, but in Paper III, 4 out of 37 subjects had impaired LTP at follow-up. The results in Paper III suggest that LTP deteriorated from baseline during the follow-up. However, no subjects had impaired LTP to the extent that protective sense was diminished, as defined by the manufacturer of Touch Test® (136, 137). In view of

the lack of reference values on normal LTP in children and adolescents, I chose to use the full 20 pieces Semmes-Weinstein's monofilament test and started with the thinnest monofilament. The discrimination levels recommended by the manufacturer were used, although these were adapted from adults. The deterioration of LTP from baseline to follow-up will be further discussed below. A study by Hirschfeld et al. 2015 studied the sensitivity and specificity of an abbreviated, three-monofilament, screening test (1 mN, 2 mN and 4 mN; roughly translated to 0.1-g, 0.2-g and 0.4-g, respectively), and of a 128 Hz tuning fork in relation to an abnormal NCS in children and adolescents with T1D aged 6-18 years (115). In that study, monofilament and tuning fork screening were performed on the foot, but the location was not specified. In conclusion, Hirschfeld et al. 2015 showed that 43 out of 88 participants had at least one abnormal NCS, but screening sensitivity and specificity was 18% and 80%, respectively, for the three-monofilament test and 0% and 98%, respectively, for the 128 Hz tuning fork (115). This lack of sensitivity with conventional screening methods calls, in my opinion, for an update of recommendations including a thorough groundwork at establishing age- and gender-specific normative values for screening.

Screening using vibrotactile sense

Screening of vibrotactile sense for the detection of DPN has been used extensively, but the use of a typical 128 Hz tuning fork is limiting. It is not graded and will thus only give a result of perceivable or non-perceivable sense to 128 Hz. It has been suggested that vibrotactile sense should be studied using VPTs instead (138), providing a value that is comparable over time and can tell us if the VPTs are increasing or decreasing. Examples of tools providing VPTs are biothesiometers, often with a fixed frequency. However, tools, where the frequency can be adjusted, like the Neurothesiometer, have also been developed and are currently used. Furthermore, graded tuning forks, like the Reidel-Seiffer tuning fork with frequencies of 64-128 Hz, exist (139, 140). Today, there are numerous manufacturers of biothesiometers and a comparison between different devices has been shown reliable in adult subjects with diabetes (141). Biothesiometers use frequencies around 50 Hz (142) or 100 Hz (143), and the intensity of the probe can be adjusted. Thus a cut-off, i.e. a VPT, can be determined to what voltage the subject perceives the vibration. In a study by Kästenbauer et al. 2004, on 2022 subjects with diabetes, the use of a Rydel-Seiffer tuning fork was suggested to be comparable to a neurothesiometer to find signs of DPN (144). However, the results from the tuning fork examination were used to claim DPN (144). Neurothesiometer data is only presented on groups with "normal" and "abnormal" sense depending on tuning fork abnormalities, with higher VPTs among subjects with an abnormal tuning fork examination compared to normal tuning fork examination, which is not surprising (144).

The use of a tuning fork or a biothesiometer to screen for DPN in children and adolescents with T1D has been reviewed in a study by Hirschfeld et al. 2014, concluding that the diagnostic accuracy of the tuning fork is very limited, but might be slightly better for the biothesiometer (63). However, the review consists of data from only five original studies: three studying tuning forks and two studying biothesiometers vs NCSs (63). Screening for DPN with tuning forks was done by Blankenburg et al. 2012 (60), resulting in a sensitivity and specificity of 19% and 87%, respectively, and by Gallai et al. 1988 (145) with a sensitivity of 3% and specificity of 98% (63). Tuning forks were also used in the study by Hyllienmark et al. 1995, examining 75 young T1D subjects with NCSs from median, peroneal and sural nerves, but sensitivity and specificity of the tuning fork method was not reported (124). The better sensitivity and specificity of the biothesiometer, compared to the tuning fork, might be explained by the use of different frequencies, or perhaps that it may be easier to determine the threshold for vibrotactile sense with a biothesiometer than with a tuning fork. The fact that especially low frequencies, like 8 and 16 Hz, were abnormal in Paper I calls for a need of a better screening tool than the tuning fork vibrating at 128 Hz.

Hirschfeld et al. 2014 suggests a slightly better diagnostic accuracy for the biothesiometer compared to a typical tuning fork when screening for DPN among children and adolescents with T1D (63). A study by Davis et al. 1997 used a 100 Hz biothesiometer for screening of DPN, suggesting a sensitivity and specificity of 82% and 75%, respectively (146). In that study, NCSs were performed on 21 subjects with abnormal VPTs and on 15 subjects, matched for height, age and disease duration, with diabetes and normal VPTs, in order to study the presence of abnormal NCSs among subjects with impaired and normal vibrotactile sense (146). In my opinion, it would be better to start with NCSs on all subjects and thereafter, through a blinding process, examine the subjects with a biothesiometer as well, since this will also reveal abnormal NCSs in the normal material. Additionally, I find the study groups in Davis et al. 1997 rather small, and the method for biothesiometer testing is quite vaguely described which makes it difficult to draw conclusions on sensitivity and specificity. For example, Davis et al. 1997 states that "VPTs were measured in a standardized fashion" and that the handheld biothesiometer was held "with a constant and firm pressure" to the examined site (146). However, I find it very hard, or in fact impossible, to apply a constant pressure to the investigated site if you are also responsible for adjusting the voltage or noting the VPT from the biothesiometer examination. Therefore, using a Vibrosense Meter, as done in Papers I and III, is a better option, since the influence of the examiner on the result is minimized. Furthermore, the examined subject is self-reporting on their perception to vibrotactile sense, which results in a VPT determined by the subject being examined and not by the examiner. This can be compared to audiometry (i.e. a hearing test), where the subject sits in a secluded room and self-reports their hearing at different frequencies. Another positive of the Vibrosense Meter in research, and clinical settings, is that the standardized examination procedure reduces the risk of

diverging results due to different examiners, who may conduct the examination differently.

Despite the flaws of the typical 128 Hz tuning fork, it is widely used and recommended in clinical guidelines for screening of DPN in both children/adolescents and adults (24, 66, 67). However, its use has been questioned regarding diagnostic accuracy of impaired vibrotactile sense in subjects with diabetes compared to the biothesiometer (147, 148). Furthermore, its use is limited not only because of the difficulties to set a reliable VPT, but also since the device is handheld and dependent on the examiner to elicit the same pressure every time. This risk is also apparent with the biothesiometer, since the probe is attached to a handheld unit. Thus, there is a need for a standardized screening tool minimizing errors dependent on the examiner. The MFV approach, used in Papers I-III, is a better option than a biothesiometer, since it can test several frequencies in a true standardized way with minimal influence from the examiner on the examination since the device is not handheld. The examined extremity is instead rested on a stand equipped with a meter that always assures an appropriate pressure on the probe.

MFV as a screening tool for DPN

Screening of vibrotactile sense for detection of DPN among adult subjects with diabetes, using a multi-frequency approach (i.e. the Vibrosense Meter used in Papers I and III of this thesis), has previously been proposed by Nelander et al. 2012 (72). Nelander et al. 2012 examined 37 subjects with diabetes (T1D: n = 30; T2D: n = 7), with a median [interquartile range] disease duration of 30 [16.5] years and compared the results of MFV with age- and gender-matched controls (72). The conclusion from Nelander et al. 2012 was that VPTs on the foot were elevated among adult subjects with diabetes compared to controls on MTH1 and MTH5 at 8, 16, 32 and 64 Hz, but not at 125 Hz (72). That is in accordance with my results from Paper I, which showed deterioration of VPTs at especially low frequencies. Another multi-frequency approach was used by Drechsel et al. 2021 studying vibration perception at only two frequencies (i.e. 30 and 200 Hz) in subjects with diabetes (mainly T2D), with and without DPN, and healthy controls (149). They found that subjects with DPN had significantly higher VPTs than healthy controls and subjects with diabetes without signs of DPN, at especially low frequencies (30 Hz). This is in accordance with Nelander et al. 2012 (72), although Drechsel et al. 2021 also observed increased VPTs at high frequencies (i.e. 200 Hz) among subjects with DPN (149). In adults with T1D, examined with the Vibrosense Meter, Lindholm et al. 2019 showed that impaired vibrotactile sense, i.e. elevated VPTs, at low frequencies was a better indicator for risk of foot ulcer development than elevated VTPs at high frequencies (150). Furthermore, they showed that VPTs at 125 Hz were elevated earlier than VPTs at low frequencies and that VPTs at 125 Hz were the only impaired ones in subjects with short disease duration (150), which is somewhat contradictory to my results from Paper I, showing mainly alterations at

low frequencies in young T1D subjects. The examination by MFV has previously been used not only on feet, but also on finger pulps. In a study by Dahlin et al. 2013, VPTs were measured at index and little fingers on subjects with long standing (>20 years) diabetes (T1D and T2D) and healthy controls, concluding that VPTs were higher at low frequencies (8 and 16 Hz) compared to healthy controls (151). In conclusion, considering the results of abovementioned studies on adults with T1D and T2D, there is evidence to support the use of a multi-frequency approach when screening for impaired vibrotactile sense, especially when looking for early signs of DPN, which is the case for children and adolescents with T1D. It has also been established that vibrotactile sense can be studied using MFV on both hands and feet. MFV is non-invasive, harmless and can be used to determine impaired vibrotactile sense among subjects with diabetes compared to controls. Thus, I believe that it is a suitable screening tool to use on children and adolescents with T1D.

Fluctuations of nerve function

In Paper I, a total of 13 out of 72 subjects presented with at least one pathological MFV examination on the foot. Looking at the data from a group perspective reveals that VPT z-scores [calculated based on previously collected reference data (103)] at low frequencies (8 and 16 Hz) were more elevated than at higher frequencies (32, 64 and 125 Hz), possibly indicating an earlier loss of function at low frequencies. At follow-up a median of 30 months later (presented in Paper III), VPTs had improved at low frequencies on the foot, especially at 16 Hz. This was surprising and raised a lot of questions regarding potential fluctuations in the vibrotactile sense over time, since HbA1c did not improve during follow-up. It was especially surprising since the number of subjects with impaired LTP increased from none to four subjects. However, in Paper III none of the subjects with impaired LTP were unable to perceive light touch from the regularly used 10-g monofilament.

It has been widely accepted that increased disease duration is a risk factor for deteriorating peripheral nerve function in T1D (2, 152, 153). However, deteriorating nerve function with increased duration of disease was not seen in Paper III compared to Paper I. This is a bit surprising, but several previous studies report that the function of peripheral nerves may improve, or at least that deterioration may be halted, over time in paediatric and young adult subjects with T1D (123, 134, 154-156), especially following tight glucose monitoring (157). Donaghue et al. 1996 followed adolescent T1D subjects, with a mean disease duration of 7 years at baseline, prospectively over 5 years and detected more abnormal peripheral nerve parameters at baseline compared to follow-up unrelated to glycaemic control (154). This is in accordance with the results of Paper III, but the subjects in Donaghue et al. 1996 (154) had higher HbA1c values than the subjects in Paper III. Moreover, Solders et al. 1997 studied newly diagnosed T1D subjects prospectively over 10 years and showed that NCS parameters deteriorated over time, but sensory

conduction velocities in the median and sural nerves initially improved within the first two years (156). This suggests that different nerves may respond differently to external attributes. Similarly, Lee et al. 2010 followed newly diagnosed T1D subjects for 5 years and suggested that all NCS parameters in motor and sensory nerves deteriorated over the follow-up period, except for in the sural nerve (123). In my opinion, a downside of many studies on DPN in children and adolescents is that subjects with a short disease duration were excluded from participating (116, 128, 133, 158). This potentially underestimates the number of subjects with early signs of DPN that might experience improved nerve function within the first years of T1D. I wanted to include subjects with T1D with a wide range of disease duration, without excluding subjects that were newly diagnosed. Thus, I did not exclude subjects with a disease duration shorter than one year. I believe that including all kinds of subjects with T1D from the outpatient clinic will better reflect the natural development of DPN over time, regardless of metabolic control early in the disease. As mentioned before, it has been stated that targeting normoglycaemia in subjects with T1D may improve nerve conduction and halt the development of clinical DPN (88, 159). Furthermore, evidence from the DCCT study suggests that previous intensive insulin treatment targeting normoglycaemia may reduce the prevalence of DPN 13 years after intensive treatment (160). In cases of insufficient metabolic control early in the course of T1D, it would be interesting to see if vibrotactile sense is impaired, and whether potential impairments vanish when a better metabolic control is achieved. In future studies on DPN among children and adolescents with T1D, I believe that it would be important to include subjects regardless of age, disease duration or metabolic control, as was the case in Papers I and III.

In my thesis, as described above, the vibrotactile sense improved over time from baseline (Paper I) to follow-up (Paper III), while LTP deteriorated. I believe that this may be a coincidence, but the study group at follow-up is too small to draw such conclusions. A plausible explanation to improved vibrotactile sense at follow-up could be a learning effect, due to repeated measurements of vibrotactile sense using MFV. However, this has been studied for the Vibrosense Meter by Ekman et al. 2020 on healthy controls, concluding that possible learning effects, resulting in lowered VPTs, was present during a six month interval with multiple examinations tight after each other, but diminished after six months without examinations (161). Similarly, repeated measurements of VPTs using other vibrometers have shown promising reliability (162). Conversely, Louraki et al. 2014 suggest a worse reproducibility of VPTs in children and adolescents with T1D compared to controls (163). However, in their study, a biothesiometer was used, and that is, in my opinion, a less reliable method, as discussed above. Furthermore, differences in vibrotactile sense and perception to light touch could be explained by the activation of different mechanoreceptors, since light touch is mainly dependent on Merkel discs and Meissner's corpuscles, while vibrations depend on the function of Meissner's and Pacinian corpuscles (49, 50, 117). This could explain why no subjects had impaired LTP in Paper I, but a few had impaired LTP in Paper III, when vibrotactile sense

improved during the same period. Although vibrotactile sense and LTP are both measures of large nerve fibre dysfunction, screening of large nerve fibre dysfunction should be conducted using at least two modalities.

Although HbA1c remained at the same level on follow-up compared to baseline, it cannot be ruled out that glycaemic variability was higher at baseline, possibly influencing nerve function at that time. To study this relationship, I suggest a prospective study, where subjects are examined at baseline and several follow-ups, and that subjects are equipped with continuous glucose monitors (CGMs) to be able to study glucose variability as a complement to long term metabolic control (i.e. HbA1c). Altogether, evidence suggests that impaired peripheral nerve function may improve over time, as in Paper III, and that the improvement can not only be attributed to improved metabolic control. In my opinion there is a need to follow subjects with T1D on a regular basis regarding signs and symptoms of DPN, to find early signs of DPN. Especially among subjects with concurrent insufficient metabolic control, since that may elicit a need for intensified treatment.

Factors associated with DPN

Several risk factors have been established for development of DPN in subjects with T1D, like metabolic control, disease duration and length of the nerve (2, 152, 164). The study by Hyllienmark et al. 1995, on young subjects with T1D, showed that nerve dysfunction correlated with increased height and (mainly long-term) poor metabolic control (124). However, correlations were also seen between nerve dysfunction and higher age at disease onset, which was also associated with increased height (124). A ten-year prospective follow-up study by Hajas et al. 2016 on children and adolescents with T1D, examining the development of DPN, showed an increase in prevalence of DPN from 24% at baseline to 63% at follow-up, with poor metabolic control being the most significant risk factor (122). In Paper I, I did not see any associations between impaired vibrotactile sense and metabolic control (i.e. last HbA1c and one-/two-year mean HbA1c), BMI or gender. However, in Paper I the subjects with impaired vibrotactile sense had a higher age at disease onset, which correlated with height, compared to the subjects with normal vibrotactile sense. This is in line with the findings by Hyllienmark et al. 1995 (124). However, height was not independently associated with impaired vibrotactile sense. I also chose to study the association of BMI z-scores in relation to impaired vibrotactile sense, but no such associations were found. BMI z-scores are calculated based on normative values from healthy children and adolescents (165, 166). The association of impaired vibrotactile sense was also studied in Paper III, but no associations were found between impaired vibrotactile sense and HbA1c, height, BMI or disease duration at follow-up. A possible explanation of the lack of association between impaired vibrotactile sense and metabolic control among our subjects is that they are in general very well managed in terms of HbA1c values. In

Paper I and Paper III, the medians [quartiles] of last HbA1c among all subjects were 57 [50-62] and 53 [50-65] mmol/mol, respectively, which is close to goal values suggested by both ADA and ISPAD (i.e. < 53 mmol/mol) (66, 167). This could also potentially influence the prevalence of impaired vibrotactile sense in Papers I and III. For example, in studies on subjects with T1D of comparable age to the subjects in Paper I, the subjects in Nelson et al. 2006 (116) (57% prevalence of DPN; NCS verified) had a one year mean HbA1c of 75 mmol/mol. In Louraki et al. 2016 (29) (34% of the subjects had at least one abnormal nerve conduction parameter), the one year mean HbA1c was 66 mmol/mol. This indicates that the prevalence of DPN might be higher among subjects with insufficient metabolic control, but also that DPN may also be present among subjects where the metabolic control is close to the recommendations, although to lesser extent (i.e. lower prevalence).

In situations of excess glucose in the bloodstream, glucose, and other monosaccharides, binds to haemoglobin, forming glycated haemoglobin. The extent to which haemoglobin has been glycated can be measured as HbA1c in a simple blood sample. HbA1c reflects a mean of blood glucose over the last weeks to months and therefore has several limitations in mirroring the long-term glycaemic environment. Hence, in Paper I, I chose to present the last HbA1c, the one-year mean, as well as the two-year mean of HbA1c to study the glycaemic control over time. In Paper I, these parameters were very similar, further suggesting that our cohort represents very well managed subjects with T1D. Another downside of HbA1c is that it is not conveying any information on glycaemic variability. In the DCCT study, examining intensive vs conventional treatment on subjects with T1D, Lachin et al. 2008 concludes that HbA1c accounts for a great majority of risk of developing microvascular complications between the two study groups (i.e. intensive vs conventional treatment) (168). However, Lachin et al. 2008 also suggests that some risk may be attributed glycaemic variability (168). In a study by Monnier et al. 2006 on subjects with T2D compared to age- and gender-matched controls, oxidative stress, measured as 24-hour urinary excretion of a specific prostaglandin, was significantly higher among subjects with T2D compared to controls (169). In their study, Monnier et al. 2006 showed that the levels of the studied prostaglandin were strongly associated with “mean amplitude of glycaemic excursions”, defined as the mean of glucose levels higher than one SD from the daily mean (170), while no correlation was seen with HbA1c (169). Similarly, Issar et al. 2020 suggested that increased glucose variability was associated with impaired nerve function in peripheral nerves and morphology in the corneal nerve (171). In Paper I, impaired vibrotactile sense was more common among subjects with insulin administration using MDI compared to subjects with CSII. I believe that the use of CSII instead of MDI may lead to less glycaemic excursions, but potentially less glycaemic variability may to a large extent also be attributed to the use of CGMs. It would have been interesting to study glucose variability and its association with vibrotactile sense in our cohort, but such data was not possible to retrieve. However, in future studies of the vibrotactile sense using MFV data from CGM should be

included. Decreased glucose variability in adult T1D subjects, with good glycaemic control, treated with CSII compared to subjects with MDI has been suggested by Bruttomesso et al. 2008, without an increase in hypoglycaemic events (172). However, conflicting results have been reported on the superiority of CSII vs MDI regarding glycaemic variability in young subjects with T1D (173, 174). To sum it all up, regardless of choosing CSII or MDI, I believe that the treatment with the fewest glycaemic excursions should be favourable for the peripheral nerves, especially regarding the results of Monnier et al. 2006 (169), but caution must be taken to minimize hypoglycaemic events.

Autoantibodies and associations with impaired vibrotactile sense

Paper II aimed to study the association of impaired vibrotactile sense and islet autoantibodies of T1D. Subjects from Paper I with data on autoantibodies to GAD65 and IA-2 (70 out of originally 72 subjects) from the time of T1D diagnosis were included in this study. Of the 70 subjects included, 45 (64%) had autoantibodies to GAD65, 58 (83%) to IA-2 and 39 (56%) to both GAD65 and IA-2. I did not see any association between impaired vibrotactile sense (as defined in Paper I) and autoantibodies to GAD65, IA-2 or both. This is in slight contrast to a study by Louraki et al. 2016, where the relationship between subclinical DPN, diagnosed by abnormal nerve conductions from median, peroneal and sural nerves in 85 children and adolescents with T1D, and autoantibodies to GAD65 and IA-2 was studied (29). In that study, 34% of the subjects had an abnormal NCS and 62% had autoantibodies to GAD65, 59% to IA-2 and 42% to both. Louraki et al. 2016 measured, as opposed to Paper II, autoantibody levels in proximity to electrophysiology and concluded that no association was seen between autoantibody positivity and abnormal NCS, but that one single parameter, i.e. peroneal sensory nerve action potential, was lower among anti-IA-2 positive and anti-GAD65 positive subjects (29). Similar results were presented by Hoeldtke et al. 2000, suggesting that subjects with recent onset T1D and high titres of autoantibodies to GAD65 had lower conduction velocities than subjects with low titres of autoantibodies, although conduction velocities were within normal range (28). However, in a larger study on 285 subjects with confirmed DPN from the DCCT cohort, Hoeldtke et al. 2007 determined that autoantibodies to GAD65 was not associated with confirmed DPN (34), which is in accordance with the results in Paper II. Thus, conflicting data has been reported.

The potential association between islet autoantibodies and the development of DPN has been suggested to depend on different mechanisms. For example, high titres of autoantibodies to GAD65 have been associated with impaired metabolic control (34), a known contributor to DPN (88). Furthermore, suggestions have been made that GAD65 autoantibodies may cause direct damage to the peripheral nerve, since GAD65 is expressed in peripheral nerves (28, 32, 35). However, in Paper IV, I was not able to identify either GAD65, or IA-2, in any of the PIN biopsies. This does

not exclude their presence in the nerve biopsies but concludes that the protein intensities were below the detection level in the LC-MS/MS model.

Proteomics as a tool to study DPN

Using quantitative proteomics on whole nerve specimens from subjects with and without diabetes, represents a novel approach to study DPN. However, the present thesis does not focus on the methodological aspects of quantitative mass spectrometry, but rather its suggested future clinical implications. A proteomic approach to studying whole nerve biopsies has both advantages and disadvantages. The method does not provide a clear topographical distribution of protein intensities in the different parts of the nerve and supporting surrounding tissues. Describing the topography can be done using immunohistochemistry, but such analyses are limited to a limited number of proteins every time (56), while mass spectrometry enables identification and quantification of thousands of proteins. For example, our nerve specimen of PIN describes the proteome of axons, micro-vessels, Schwann cells and other cells around the nerve altogether. However, suppose Schwann cell specific proteins of interest to DPN could be identified, these could be studied using our quantitative proteomics approach. To the best of my knowledge, the full proteome of PIN has never been presented before, prior to Paper IV.

PIN as a model of DPN

In Papers IV and V, biopsies of PIN were used to study DPN instead of, for example, the sural nerve, which historically has been the conventional approach. Biopsy of the sural nerve, however, is associated with a range of complications (118, 119, 175). Biopsy of PIN has been demonstrated to be safe, and well tolerated by the subjects (54). Furthermore, it has previously been shown that PIN from subjects with T2D, compared to post-mortem controls, show signs of reduced myelinated nerve fibre density (54). Most of the PINs studied in Papers IV-V were harvested from subjects with concurrent CTS undergoing carpal tunnel release. However, the PIN is an uncompressed nerve on the dorsal aspect of the forearm, while the compression neuropathy of the median nerve in CTS is located on the ventral aspect. Thus, I found it safe to use PIN to study the effects of diabetes on peripheral nerves, without having to consider CTS a confounder of nerve dysfunction in PIN. A downside of PIN, however, is that it is not as distally located as the distalmost peripheral nerves in the lower extremities. Since DPN is considered a length-dependent neuropathy (2), it is possible that alterations in PIN will arise later than in, for example, the sural or peroneal nerves. On the other hand, PIN, and more distally located nerves, are exposed to the same levels of blood glucose, which argues for additional causes than solely hyperglycaemia as causes of DPN. A

possible explanation is that distal nerves in the upper extremity are better vascularised than peripheral nerves in the lower extremity, like the peroneal and sural nerves, which can be argued since previous studies indicate that endoneurial capillary densities are higher in PIN than in the sural nerve (55, 176). Another possible explanation is that the neural environment and response to hyperglycaemia is different in the proximal part of the nerves, like DRGs, compared to the distal part, which has been suggested from animal models on DPN (177).

Proteomics of PIN

The quantitative proteome of PIN has to the best of my knowledge previously never been described until Paper IV was published. Paper IV aimed to show the feasibility of using proteomics on human nerve biopsies. Several ongoing projects worldwide have engaged in creating a proteome map of the human body describing proteome differences between tissues (178, 179). In Paper IV, 2617 different proteins were identified and quantified in PIN from subjects with T2D and healthy controls. However, no clear differences were seen in protein expression between groups. One possible explanation might be that the T2D subjects are, in general, exceptionally well treated in terms of metabolic control with median HbA1c within the normal range of HbA1c in the population. Nevertheless, all T2D subjects underwent OGTTs confirming the diagnosis. The majority of T2D subjects had been diagnosed with T2D for more than 15 years, which should, though, be associated with some alterations in peripheral nerve function. Furthermore, electrophysiology parameters showed only early signs of large nerve fibre dysfunction in the T2D group. Although some of the T2D subjects had more extensive DPN, the study group was quite small and the majority of T2D subjects had normal electrophysiology. This may impact the result when groups are compared, especially since several T2D subjects had normal electrophysiology, and in some cases better nerve conductions than some of the healthy controls. However, none of the healthy controls presented with nerve conduction parameters outside normal range.

Difficulties using a proteomic approach to study PIN

A possible explanation for the lack of clear differences in protein expression between T2D subjects and healthy controls in Paper IV is associated with the challenge of big data. More than 2600 proteins were identified and quantified. This means that large differences in protein expression between groups are needed to reveal potential proteins of specific interest to DPN development, since statistical measures are taken to reduce type I errors in hypothesis testing (i.e. controlling for false discovery rate). Furthermore, since DPN development is multifactorial, with multiple co-existing pathways, and not dependent on altered gene expression of single genes, it is unlikely to find a certain protein that unravels the mystery of DPN.

However, finding proteins that contribute to the dyshomeostasis in the peripheral nerve is important since they may constitute potential drug targets. Searching for such proteins is difficult though, since differing protein expressions are not only a consequence of disease (i.e. diabetes in this case), but can also be a consequence of inter- and intrapersonal normal differences, resulting in large intergroup differences. Moreover, differences in protein expression will constantly change since the proteome is highly dynamic.

Multiple opportunities are revealed using proteomics on PIN

The great possibilities with a proteomic approach to study DPN must be highlighted. Proteomics can be used to study numerous disease pathways simultaneously and has been considered a promising tool when searching for biomarkers for disease and for complications of disease (180). Since the pathogenesis of DPN is heterogenic, it is likely that not only one biomarker of DPN should be searched for, but rather groups of biomarkers that may predict complications in different subjects, as has been discussed in previous studies (180).

In Paper IV, I could identify proteins in PIN known to be involved in, and to contribute to, a wide range of structures, complexes, processes etc. within and around the cell. For instance, in Paper IV, the PIN biopsies expressed several different collagens, known to be essential for extracellular matrix structure and remodelling, and to play crucial roles for Schwann cells regarding, for example, axonal support and myelination (181). Furthermore, several proteins essential for proper myelin formation and stabilization, like myelin protein zero and myelin basic protein (182), were identified in PIN. Moreover, numerous cytoskeletal proteins, and proteins interacting with the cytoskeleton were identified. I find this compelling, since the cytoskeleton plays key roles in axonal transport, implicated in DPN development and associated with several other neurological diseases (183, 184). In Paper IV, several proteins connected to lipid metabolism and inflammation were also identified, which is of great interest, especially regarding DPN development in T2D.

The opportunities are countless with a proteomic approach to the study of DPN. The challenge is, in my opinion, to narrow down the search of potential proteins of interest. Although I have been able to identify proteins of interest to DPN, and many of these proteins are known to contribute to outlined pathogenic pathways in DPN, the quantitative proteomic approach does not provide proof of how the proteins interact with the pathways. This needs to be further studied in studies where the topographical distribution and action of the proteins can be determined. I find it, nevertheless, valuable to use proteomics for large-scale screening for potential protein biomarkers of DPN.

Heat shock proteins in PIN and DPN development

Several different HSPs have been described to play a role in DPN development (75, 94, 185-188). As described above, HSPs react to several different types of stress to maintain protein homeostasis within the cells. Previous studies have suggested an association of DPN to HSP90 and HSP70 (75), but also to HSP27 (188, 189). Furthermore, a neuroprotective role has been suggested for HSP27 in animal models of diabetes (190). Potential drugs have been discovered and tested in animal models of T1D and T2D, suggesting that modulations of HSP70 and HSP90 may improve peripheral nerve function by, for example, improved mitochondrial bioenergetics (97, 191, 192). A study by Atkin et al. 2021 on subjects with T2D and healthy controls showed different expressions of HSPs in blood plasma between groups, and concluded that targeting euglycemia may lower the level of HSPs in plasma (193). This suggests that HSPs were overexpressed in the blood plasma due to the stress from hyperglycaemia, but that levels subsequently declined in response to normalisation of blood glucose. Altogether, there is convincing evidence that HSPs may play a role in DPN development and should be targets for further research.

In Paper V, I presented 32 HSPs (including putative HSPs) that were identified in PIN from subjects with T1D, T2D and healthy controls. No clear differences were seen regarding protein expression of HSPs between groups, but I believe that much can be explained by the same reasons discussed above; i.e. small study groups, heterogenous groups with only early signs of neuropathy, large differences in characteristics within groups etc. However, in the unadjusted linear regression model, several HSPs approached statistical significance regarding differing protein expression between groups. This suggests that significant differences in protein patterns could be apparent in a larger cohort, or in a cohort with more homogeneity (in terms of age, disease duration, abnormal NCSs etc.) and more severe DPN. Furthermore, Paper V revealed that almost all HSPs were identified in all subjects, but some HSPs were missing in a few subjects. For example, HSP beta-8 was missing in six subjects with diabetes, but was present in all healthy controls. This is interesting, since HSP beta-8 has previously been suggested to be associated with neurite degeneration in motor neuron disease (194).

In Paper V, there was a tendency for HSP27 to be more abundant in PIN from T1D subjects than in subjects with T2D and healthy controls (Figure 9). However, the results were not statistically significant. This would be interesting to study further in a larger cohort, or in a cohort of subjects with solely confirmed clinical DPN, since only some subjects with T1D and T2D in Paper V have confirmed DPN, but some have normal NCSs in all parameters. A study by Pourhamidi et al. 2014 showed that HSP27 levels in blood serum were lower in T1D subjects compared to healthy controls, and showed that lower levels of HSP27 correlated to progression of large nerve fibre dysfunction, suggesting a neuroprotective role for HSP27 (189). A neuroprotective role for HSP27 in diabetes has also been suggested from animal

studies (190). It would be interesting to study the relationship between the level of HSP27 in blood and in PIN and correlate these levels to data from NCSs. I believe that the abundancy of HSP27 within the nerve could be a better indicator of neuroprotection than the level of HSP27 in blood.

The advantage of using PIN

As mentioned above, taking biopsies of PIN is associated with minimal discomfort and no known postoperative complaints or complications (54), which suggests that its superior in terms of complications compared to harvesting biopsies of the sural or peroneal nerve (118, 119, 175, 195). A possible explanation for less post-operative complications following PIN biopsy is that the upper extremity might be more vascularized than the lower extremity (55, 176). Nevertheless, general caution must be taken when biopsies are harvested, especially from subjects with diabetes and potentially impaired wound healing and increased risk of infections.

DRGs, especially in studies on neuropathic pain, cultured Schwann cells and animal models of diabetes, and subsequent nerve biopsies (mostly sciatic nerve) have been widely used to study the pathogenesis of DPN (2, 17, 196), but issues with these approaches are evident and should be addressed. Biopsy of DRGs from animal models of diabetes to study neuropathic pain and DPN is connected to a range of potential errors. For example, animal models do not represent a comparable environment to T1D and T2D in humans, and the DRGs studied are in the most proximal parts of the peripheral nerve, while symptoms arise first in the distal part. As described by Freeman et al. 2106, the distal nerve and the proximal DRGs might respond differently to hyperglycaemic conditions (177). There are many reasons for why PIN is a promising target for DPN studies. First and foremost, since it is human nerve tissue, it reflects the true influence of T1D or T2D to peripheral nerves and it is not dependent on a model reflecting induced diabetes, like for example streptozotocin. Furthermore, it can be easily harvested, as discussed before, and can provide information not only about the neurons, but also about surrounding tissues, as presented in Paper IV and V. A clear advantage of using PIN to study DPN is that the neurotrophic support of surrounding tissues can also be studied. For instance, chances are high that the interplay between the axons and surrounding Schwann cells, known to be crucial for support and survival (197), can be studied further using proteomics. An example is that several extracellular matrix proteins were identified in Paper IV, and their interplay with Schwann cells is intriguing.

Previous studies have been engaged in determining quantitative proteome profiles of tissues or cells of interest to the peripheral nerve. For example, a proteomic approach has been used to study myelin in PNS from healthy mice and in a neuropathic mouse model, concluding that protein differences could be detected between the two models (198). This is promising in my view, since it suggests that single protein differences can be determined through a large-scale method like

quantitative proteomics, which was seen also in Paper V on some HSPs, albeit not reaching the level of statistical significance. In addition, a quantitative proteomic approach has been used to study nerve regeneration in ex-vivo nerve degeneration, and shared proteomic profiles of human peripheral nerves and Schwann cells (199). Quantitative proteomics has also been used to study nerve injury and regeneration in rats (200). In a chronic constriction injury model in rats, the use of quantitative proteomics suggested a role for the protein Annexin A3 in neuropathic pain (201). In Paper IV, I show that Annexin A3 was abundant in PIN from both T2D subjects and healthy controls, which in my opinion indicates that quantitative proteomics in animal models of disease and complications may serve as tool for highlighting proteins of interest, that could subsequently be studied further in human tissues. Since the use of quantitative proteomics is scarce in DPN studies, it has been hard to find studies that have shown similar, or contrary, results to those I have presented. However, Leal-Julià et al. 2021 conducted a study on sciatic nerves and DRGs from healthy and db/db (i.e. a commonly used animal model for T2D) mice (202). They presented the proteomes from sciatic nerves and DRGs in diabetic mice and showed that multiple proteins of interest to DPN development were either up- or downregulated. Many of these proteins were identified and quantified also from PIN in Paper IV. Interestingly, Leal-Julià et al. 2021 showed that multiple HSPs were either up- or downregulated in the sciatic nerve (202), further suggesting a role for HSPs in DPN development as I described in Paper V. In summary, there is emerging evidence, from studies of both animal and human tissues, that quantitative proteomics has a role in future research of nerve damage and repair, as well as of DPN. I strongly believe that this is a research field that will evolve considerably over the years to come, and it will surely include machine learning in the future.

Connecting genetic susceptibility and proteomics

Several genetic variants, i.e. SNPs, have been proposed to either increase or decrease the risk of DPN (132, 203-205). I believe that targeting the proteins of those genes with a proteomic approach would be interesting since those genes have been shown to have an impact on DPN development. Recent studies on blood plasma have integrated the use of GWAS with quantitative proteomics, providing a link between the genetic code and expressed proteins (206-208). However, while blood plasma is easily retrieved from subjects, nerve biopsies are much more difficult to harvest. It will therefore be important to link the proteomes of peripheral nerves and blood plasma, so that future biomarkers of DPN can be studied in blood plasma instead of peripheral nerve biopsies. Hence, there is a need for future research to study the relationship between genetic variants and similarities of proteomes from blood plasma and peripheral nerves.

Strengths and limitations

There are obvious limitations to my studies in Papers I-III that need to be addressed. The lack of NCSs and no clinical examination at the time of MFV examination makes it impossible to confirm DPN, since this requires abnormal NCSs, especially in the case of subclinical DPN (65). I believe that data from NCSs and clinical examinations would have markedly increased the impact of Papers I-III, given that MFV could be related to data from NCSs. Finding a sensitive substitute to NCS for early detection of DPN could in fact be ground-breaking, but in Papers I-III resources were limited and the availability for NCSs was insufficient. However, a strength of my studies is that the VPTs resulting from the MFV examinations in Papers I-III have been compared with a previously collected large set of normative data, which was grouped based on age and gender. The age groups of the normative values by Dahlin et al. 2015 was set to reflect pre-puberty (8-10 years), puberty (11-15 years) and post-puberty (16-20 years) (103), but it could also be argued that Tanner stage would be a better grouping variable than age intervals. Nevertheless, using normative values grouped based on age and gender is, in my opinion, a good alternative when Tanner stage is lacking. Since no clinical examination was done at time of examination, and since no ethical approval was granted for such staging, Tanner stage could not be determined.

Furthermore, the participants in my studies were not asked for signs and symptoms of DPN. This could have been done using a standardized questionnaire, like the neuropathy symptom score (142). However, this has not been validated for children and adolescents. In Paper I and Paper III, none of the subjects had any clinical signs or symptoms of DPN according to their medical records, but there is of course a risk that such information has been unreported in the records, especially since screening of DPN is not conducted in all subjects at the outpatient clinics. To reduce the risk of undiscovered symptomatic DPN, a thorough medical examination could have been carried out. A strength, however, is that children and adolescents with T1D in Sweden are closely monitored and followed-up and the medical records are of very high standards compared to several other countries around the world.

The subjects in Paper IV were meticulously monitored, which is mainly a strength but, in some aspects, also a limitation. The close study monitoring contributed most likely to improved metabolic control, which is reflected in the HbA1c values of the subjects with T2D being close to normal. Furthermore, only early signs of DPN were seen in the T2D subjects in Paper IV. This may be a limitation considering that

the proteome in mildly affected nerves of subclinical subjects may differ from the proteome in nerves from subjects with severe clinical DPN. Most likely, alterations in the proteome would be, to some extent, dependent on the severity of nerve damage.

A strength of Papers IV and V is that they include both T1D and T2D subjects, as well as healthy controls. On the downside, though, groups are not numerically equal. Skewness in data may be present since the group of T1D subjects is markedly smaller than the two other groups.

To reduce the likelihood of finding proteins not expressed in the nerve or surrounding tissue, PINs were washed after harvest to clean them from as much blood residues as possible. Despite a thorough cleanse, some blood residues will also be analysed, which may represent a limitation of using PIN, or other nerves, for DPN studies. An obvious risk of analysing PIN when a large amount of blood residue is present is that the proteomic analysis may, to some extent, reflect the protein intensities in the blood plasma and not the peripheral nerve.

A pronounced strength of using PIN as a model for DPN is of course that it is a human nerve, and truly reflects the neural environment in T1D and T2D. On the other hand, the more proximal location of PIN, compared to the sural or peroneal nerves, may be a disadvantage.

Conclusions

The present thesis describes two novel approaches to the study of DPN. The first approach is using multi-frequency vibrometry to study impaired vibrotactile sense, a sign of probable DPN, in children and adolescents with T1D. The second approach is to use quantitative mass spectrometry to describe the proteome of PIN from subjects with T1D, T2D and healthy controls. The key findings of this thesis are as follows:

- Impaired vibrotactile sense, an indicator of probable DPN, can be screened for using MFV in children and adolescents with T1D.
- Approximately 20% of the subjects showed signs of impaired vibrotactile sense, while none had impaired LTP.
- Vibrotactile sense might improve over time in children and adolescents with T1D, regardless of metabolic control, with concurrent deterioration of LTP.
- Screening of large nerve fibre dysfunction in children and adolescents with T1D should be conducted on multiple modalities, i.e. vibrotactile and light touch perception, using a multi frequency approach and an extended monofilament collection.
- Impaired vibrotactile sense was more common among subjects receiving insulin through MDI compared to CSII.
- No associations were seen between impaired vibrotactile sense and autoantibodies to GAD65, IA-2 or both, from time of clinical T1D onset.
- Quantitative proteomics represents a novel approach to study whole nerve biopsies from adult subjects with T1D, T2D and healthy controls.
- More than 2600 different proteins were identified in PIN from adult subjects with T1D, T2D and healthy controls.
- A total of 32 known and putative HSPs were identified in PIN.
- No associations were seen between protein expression and diabetes or NCS parameters.

Future perspectives

Screening of DPN in children and adolescents with T1D

Screening of DPN should start early among paediatric subjects with T1D, but the optimal time to start screening is yet to be established. Future research on DPN among children and adolescents should include prospective studies with multiple screening tools and subjects should, in my opinion, be included at time of clinical T1D onset. In that way, natural fluctuations of large nerve fibre function in the adapting and developing child may be elucidated. Hopefully, this will result in increased screening for DPN in young T1D subjects, and I suggest that results from screening are reported to national diabetes registers, enabling DPN research from large cohorts in the future. Furthermore, the impact of puberty on DPN development may be further addressed. In my opinion, future research on DPN in paediatric subjects should also include glucose variability, obtained from CGMs, as a parameter of metabolic control. It is about time that the use of a single 10-g monofilament for testing LTP, and a 128 Hz tuning fork for testing VPTs, is studied in comparison with MFV, using parameters from NCSs as the gold standard defining DPN. Such studies should also include thorough clinical examinations and questionnaires asking for signs and symptoms of clinical DPN.

Proteomics of peripheral nerves in DPN

The use of quantitative proteomics to study DPN in subjects with T1D and T2D is in my opinion promising. I believe that it will be especially useful on cohorts where subjects have signs of severe DPN, as opposed to the subjects in my studies, and in studies where hypotheses are clearly defined. A more thorough study of HSPs in peripheral nerves would be especially interesting. Furthermore, proteomics can be used to study key proteins of previously described pathogenic pathways of DPN. Future studies should also apply the use of quantitative proteomics to nerve biopsies from the lower extremity, like the sural nerve, and a comparison of the proteome from different nerves would be beneficial. Hopefully proteomics can aid in the search for biomarkers or drug targets of DPN. I strongly believe that the use of quantitative proteomics will massively increase in clinical research, with help from machine learning.

Populärvetenskaplig sammanfattning

Diabetes är inte en enskild sjukdom, utan en rad tillstånd som påverkar kroppens förmåga att reglera nivån av socker (dvs. glukos) i blodet. De två vanligaste typerna av diabetes är typ 1-diabetes och typ 2-diabetes. Typ 1-diabetes beror på att kroppens förmåga att producera hormonet insulin har försämrats eller helt slagits ut, på grund av en autoimmun reaktion, dvs. att kroppen själv angripit delar av bukspottskörteln. Insulin är ett hormon som produceras i bukspottskörteln och som bistår kroppen att ta upp glukos i olika vävnader. Typ 2-diabetes däremot beror på flera faktorer, där en försämrad omsättning av glukos i kombination med nedsatt känslighet för insulin är centrala orsaker. Typ 2-diabetes är också tätt sammankopplat med övervikt och fetma.

Både typ 1- och typ 2-diabetes är sjukdomar som är förknippade med flera följdsjukdomar, även kallade komplikationer. Några av de vanligaste komplikationerna till diabetes är påverkan på ögon (s.k. diabetesretinopati), njurar (s.k. diabetesnefropati) och nerver (s.k. diabetesneuropati). De nerver som oftast påverkas av diabetes är så kallade perifera nerver, alltså de nerver som styr muskler (motoriska nerver), bland annat till armar och ben, och de nerver som tar emot signaler från omvärlden (sensoriska nerver), i fråga om känsel. Skador på motoriska och sensoriska perifera nerver brukar kallas för perifer diabetesneuropati.

Perifer diabetesneuropati är en vanlig komplikation till både typ 1- och typ 2-diabetes och saknar behandling. Komplikationen är förknippad med stora besvär för den som drabbas, både i form av smärta och risk för fotsårsutveckling samt i värsta fall amputation. Eftersom effektiva behandlingar saknas tas stora resurser i anspråk av sjukvården för vård av personer med perifer diabetesneuropati. Komplikationen är väl studerad, och flera olika orsaker till den har beskrivits, men trots detta saknas effektiv behandling. Flera av orsakerna till perifer diabetesneuropati är förknippade med att blodsockernivån hos personer med diabetes är förhöjd och ofullständigt reglerad. Tidigare forskning visar även att barn och ungdomar med typ 1-diabetes ofta visar tecken på perifer diabetesneuropati, utan att uppvisa några symptom.

Denna avhandling är uppdelad i två fokusområden, där det första syftar till att leta efter tidiga tecken på, även kallat att screena för, perifer diabetesneuropati hos barn och ungdomar med typ 1-diabetes genom att undersöka deras vibrationskänsel i händer och fötter. Det andra fokusområdet syftar till att beskriva vilka

äggviteämnen, s.k. proteiner, som förekommer i perifera nerver hos personer med typ 1-diabetes, typ 2-diabetes och hos friska personer.

Screening av perifer diabetesneuropati hos barn och ungdomar med typ 1-diabetes

I den första delen av avhandlingen används en screeningmetod, så kallad multifrekvensvibrametri, för att studera känseln för vibrationer i händer och fötter hos barn och ungdomar med typ 1-diabetes. Vibrationskänseln brukar användas för att studera tecken på perifer diabetesneuropati. Multifrekvensvibrametri är unikt för att den testar känseln för flera olika frekvenser på ett standardiserat sätt. På så vis är förhoppningen att avvikande känsel ska kunna upptäckas tidigare jämfört med om endast en frekvens studeras, vilket är det brukliga i dagens diabetesomhändertagande. Genom multifrekvensvibrametri visade 13 av 72 (18%) undersökta barn och ungdomar tecken på nedsatt känsel för vibrationer. Jag kunde även visa att känseln över tid sannolikt kan förbättras och försämras. Därtill studerades nedsatt vibrationskänsel i relation till autoantikroppar, dvs. potentiellt skadliga proteiner som bildats mot kroppen själv, som är vanligt förekommande vid typ 1-diabetes, men några sådana associationer kunde inte ses.

Proteinprofil av perifera nerver vid perifer diabetesneuropati

I avhandlingens andra del togs nervbitar från personer med typ 1-diabetes och typ 2-diabetes, samt från personer utan diabetes. Dessa nervbitar, från nervus interosseous posterior belägen strax ovan handleden, studerades ingående avseende vilka proteiner de innehåller, där så kallad mass-spektrometri och kvantitativ proteomik användes. Jag kunde visa att minst 2617 olika proteiner finns representerade i nerverna. Jag kunde inte se några skillnader i proteinprofil mellan personer med och utan diabetes. Nervfunktionen hos personerna som ingick i studien hade tidigare undersökts. I avhandlingen kunde jag inte se några tydliga kopplingar mellan nedsatt nervfunktion och proteinprofil.

Slutsats

Avhandlingen presenterar två nya sätt att studera perifer diabetesneuropati, dels genom screening av vibrationssinnet hos barn och ungdomar med typ 1-diabetes, dels genom att beskriva proteinprofilen i en perifer nerv. Min förhoppning är att screening av perifer diabetesneuropati i framtiden ska innefatta multifrekvensvibrametri samt att framtida studier av uppkomsten till perifer diabetesneuropati fokuserar på de proteiner som finns representerade, och möjligen kan vara förändrade, i nerven.

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Conflict of interest

I have no conflicts of interest to declare. I am neither affiliated, nor financially associated, with Vibrosense Dynamics AB.

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Paper I

PLOS ONE

RESEARCH ARTICLE

Impaired vibrotactile sense in children and adolescents with type 1 diabetes – Signs of peripheral neuropathy

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Abstract

Objective

To investigate whether multi-frequency vibrometry can identify individuals with elevated vibration perception thresholds (VPTs), reflecting impaired vibrotactile sense, among children and adolescents with type 1 diabetes.

Methods

In 72 pediatric patients with type 1 diabetes, VPTs were evaluated for seven frequencies on two sites of the hand, and five frequencies on two sites of the foot. Z-scores, based on previously collected reference data, were calculated. Perception to light touch was investigated using monofilaments. Subjects' characteristics were analyzed in comparison to normal and impaired vibrotactile sense.

Results

Subjects' median age, disease duration and age at disease onset were 12.8, 5.3 and 6.9 years, respectively. A total of 13 out of 72 (18%) subjects had impaired vibrotactile sense on at least one foot site. Impaired vibrotactile sense was more common among subjects treated with multiple daily insulin injections (MDI) compared to subjects treated with continuous subcutaneous insulin infusion (CSII) ($p = 0.013$). Age at disease onset was higher among subjects with impaired vibrotactile sense ($p = 0.046$). No significant correlations were found with gender, HbA1c or duration of diabetes.

Conclusions

Impaired vibrotactile sense, mirroring diabetic peripheral neuropathy, was found in 1/5 of the children and adolescents in the study, and was more common in patients treated with MDI than in subjects treated with CSII.

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Abbreviations: BMI SD, BMI standard deviation score; DPN, Diabetic peripheral neuropathy; LTP, Light-touch perception; MTH 1, First metatarsal head; MTH 5, Fifth metatarsal head; VPT, Vibration perception threshold.

Introduction

Peripheral neuropathy is a well-known complication to diabetes type 1 and 2 (T1D; T2D), that not only affects the patients' physical health and ability to be physically active, but also influences their quality of life [1–3]. Furthermore, diabetic peripheral neuropathy (DPN) is the most important factor in the development of diabetic foot ulcers–a major health and economical issue worldwide [4–6]. A recent study estimated the cost of diabetic foot care in the UK in 2010–11 to £580 millions [7]. Although we do not have any cure for DPN today, it is important to find the subjects with DPN, in order to increase awareness that these patients are likely to develop diabetic foot ulcers in the future.

Children and adolescents have also been shown to, at least in cases of unsatisfying metabolic control, present with signs of DPN [8, 9]. Although these signs are visible, most of these children and adolescents are asymptomatic and the DPN is thus in a subclinical stage. When using electrophysiology, i.e. nerve conduction study, on children and adolescents, abnormalities have been detected in 28–58% of the cases [10]. The nerve conduction studies are the gold standard method for the diagnosis of DPN. However, they might be invasive, time consuming and not always easy to access. Thus, there is a need of accessible and sensitive screening methods for the early diagnosis of DPN [11, 12]. Measuring perception to vibrotactile stimulus with tuning forks or a biothesiometer and perception to light touch (LTP) with multiple monofilaments have been suggested as suitable methods [13]. However, studies testing these methods have given inconclusive results with widely varying sensitivity and specificity in comparison to nerve conduction studies [9, 10]. An article by Louraki et al. reported that screening for DPN using a biothesiometer resulted in a prevalence of abnormal vibrotactile sense varying between 6.2 and 62.5% between different studies [9]. The great difference in prevalence is thought to depend on different cut-off points, as well as, different definitions of diabetic neuropathy. An important issue with using a biothesiometer in screening for DPN is that the reproducibility is limited in comparison to nerve conduction studies; Louraki et al. report that the reproducibility was lowest among the subjects with the longest disease duration, the poorest metabolic control, as well as the presence of obesity [14]. Today, consensus is lacking on when to start, and how to perform, screening for DPN in children and adolescents [15].

The vibrotactile sense depends on the function of Meissner´s and Pacini´s corpuscles in the skin, reflecting large nerve fiber function. Using the vibrotactile sense to look for alterations mirroring impairments in the function of large nerve fibers is well established, and furthermore, the foundation of screening methods, such as tuning forks and biothesiometers [16]. Meissner´s corpuscles are most sensitive to frequencies around 30 Hz [17] and Pacini´s react mainly to vibrations around 150–250 Hz [18, 19]. With this in mind, a method stimulating both of these vibrotactile receptors would, at least theoretically, provide a more truthful picture of the status of vibrotactile sense in the glabrous skin. A previous study using the Vibrosense meter for the examination of vibration perception thresholds (VPTs) in the hands of adults with T1D in comparison with age and gender matched controls has shown that patients, 20 years after diagnosis, had higher VPTs in the hands, mainly at low frequencies, than controls [20]. The Vibrosense meter has several advantages compared to tuning forks and a biothesiometer, for example being user dependent instead of examiner dependent. The Vibrosense meter runs an examination depending on the response of the subject being examined, whereas a biothesiometer is dependent on the one holding the instrument. In order to use a biothesiometer or a tuning fork, the examiner needs to hold the device with a constant pressure to the skin. The Vibrosense meter on the other hand, gives an instant report, by turning on and off different led lights, when the subjects pressure on the vibrating probe is too hard or too soft [21].

There are no previous studies using the Vibrosense meter in children and adolescents with type 1 diabetes. However, there is a study on healthy children and adolescents representing the normal material of this study [21]. Furthermore, studies using the Vibrosense meter in adults have shown higher VPTs in adults with T1D and T2D compared to controls [22].

Our aim was to evaluate VPTs, obtained with a VibroSense Meter, and LTP, using Semmes-Weinstein's monofilaments, in children and adolescents with T1D, in order to investigate if subjects with impaired sense, reflecting underlying sensory DPN, can be identified in relation to previously collected normative data [21]. We also aimed to investigate epidemiologic and clinical factors associated with impaired vibrotactile sense.

Subjects and methods

Subjects

Children and adolescents with T1D at the pediatric outpatient clinics at Skåne University Hospital and the Hospital of Helsingborg were, between April 2015 and June 2016, when visiting their pediatrician for regular follow up of their diabetes, asked for participation in this study. The regular follow up includes assessment of HbA1c values, measuring height and weight, and targeted physical examinations based on symptoms. Inclusion criteria: all patient interested in participating and diagnosed with T1D. Exclusion criteria: younger than eight or older than 18 years of age, subjects with non-analyzable curves, as well as subjects with other diseases than T1D, coeliac disease or autoimmune thyroiditis than can also give symptoms of impaired vibrotactile sense.

Eighty-two children and adolescents (boys = 43) accepted the invitation. Four subjects (boys = 2) did not meet the age criteria and five subjects (boys = 2) were excluded due to non-analyzable curves. One patient was excluded due to concomitant juvenile arthritis, treated with methotrexate. Eight patients (11%) suffered from concomitant celiac disease and six patients were having thyroid autoantibodies (8%), but only three of these (4%) where treated with levothyroxine. Furthermore, one of the subjects participated in the Etanercept Diamyd Combination Regimen study (ClinicalTrials.gov Identifier: NCT02464033). None of the included patients suffered from chronical diseases other than T1D, celiac disease or autoimmune thyroiditis. No subjects were excluded from participation or statistical analyses if they participated in other studies, or if they suffered from concomitant celiac disease or autoimmune thyroiditis.

The local ethics committee at Lund University approved the study (386/2007). Written informed consents were obtained from the legal guardian(s) of the children and adolescents participating in the study, as well as from the participants themselves. The research is conducted in accordance with the Declaration of Helsinki.

Methods

All examinations were carried out by two research nurses, as described below and previously reported [21]. In half of the patients, VPT and LTP measurements were performed initially in the hand, followed by the foot and in the other half, measurements were performed in the opposite order, starting with the foot, with the intention to adjust for possible lack of compliance at the end of each examination.

Vibration perception thresholds. In short, VPTs are obtained by letting the subject push a button when vibrations, from a vibrating probe, are perceived on the hand or foot, and release the button when the vibration is no longer noticed. This results in curves with several endpoints, reflecting the button being pushed and released, as well as numerical values of the vibrotactile thresholds, for all frequencies and sites tested.

Examinations were performed in a secluded examination room, and the patients wore hearing protectors in order to maintain a calm and quiet environment [21]. VPTs were obtained from the finger pulps of index and little fingers in the right hand, reflecting the median and ulnar nerves respectively, at seven frequencies (8, 16, 32, 64, 125, 250 and 500 Hz), using a standard VibroSense Meter device (VibroSense Dynamics AB, Malmö, Sweden) [21]. On the foot, the measurements were done from the sole at the first and fifth metatarsal heads (MTH 1; MTH 5) of the right foot, reflecting the function of the medial and lateral branches of the tibial nerve, at five frequencies (8, 16, 32, 64 and 125 Hz) using a modified VibroSense Meter (VibroSense Dynamics AB, Malmö, Sweden) adopted for measurement on the feet [21].

Z-scores for the numerical results of VPT assessments were calculated in comparison to previously collected normative data [21]. The resulting curves from the vibrometry examination were manually studied and visually abnormal frequencies were removed prior to statistical analyses. To be included for statistical analysis, each subject needed to present at least one visibly correct site, out of the four sites tested. Furthermore, each site being included for statistical analysis needed to have at least three visibly normal frequencies, i.e. the VPT curve needed to have at least five endpoints and the pattern needed to be structured, with no outlying endpoints reflecting lack of concentration. Examples of visibly incorrect vibrograms are shown in S1 Fig, and visibly correct vibrograms are shown in Fig 1. A site was considered pathological when at least three of the frequencies had z-scores of >1.96.

Perception to light touch. Using a 20 pieces Semmes-Weinstein's monofilament collection [Touch-Test™, North Coast Medical Inc., Morgan Hill, Ca, USA; filaments ranging from 1.65 (0.008 g) to 6.65 (300 g)], LTP was assessed by applying the thinnest monofilament, size 1.65, with a firm, constant pressure to the same sites as VPTs were measured. If the subject was not able to perceive the stimulus the procedure was repeated with the next, thicker monofilament. This method was repeated until a positive response was achieved. In the hand, tactile sensitivity to ≤ 2.83 (0.07 g) were considered normal, and for the plantar surface of the foot normal tactile sensitivity was considered ≤ 3.61 (0.4 g), as recommended by the manufacturer, but lower than previously used thresholds in a study on pediatric subjects by Nelson et al. [23]

Statistical analyses. Data are presented as medians and quartiles for the entire study group, and for boys and girls, respectively. The study group was split into two groups, based on median disease duration [11]. Differences in characteristics and obtained VPT values between the groups were tested with non-parametric Mann-Whitney U-tests. When comparing VPT values, a Bonferroni correction was applied due to a large number of analyses (k = 24).

Correlations between subjects presenting with one or more sites considered pathological, due to previously stated criteria, and different characteristics of the subjects were performed using Chi2 test. All statistical analyses were made using IBM SPSS Statistics (Statistical Package for the Social Sciences, SPSS Inc., Chicago, Il, USA) version 23 for Mac.

Results

Subjects

Listed in Table 1 are the characteristics of the 72 children and adolescents (boys = 39) meeting the inclusion criteria. Median age was 12.8 [11.5–15.0] years and median disease duration was 5.3 [2.9–8.6] years. Median age at disease onset was 6.9 [4.7–10.3]. The median of last HbA1c values, prior to examination, among the subjects was 7.3 [6.7–7.8]% (57 [50–62] mmol/mol). A total of 45 subjects were treated with continuous subcutaneous insulin infusion (CSII) and 27 subjects were given insulin as multiple daily injections (MDI). Presented in Table 1 are also the characteristics for the two groups based on gender and median split of disease duration; i.e. less than, and more than 5.3 years.

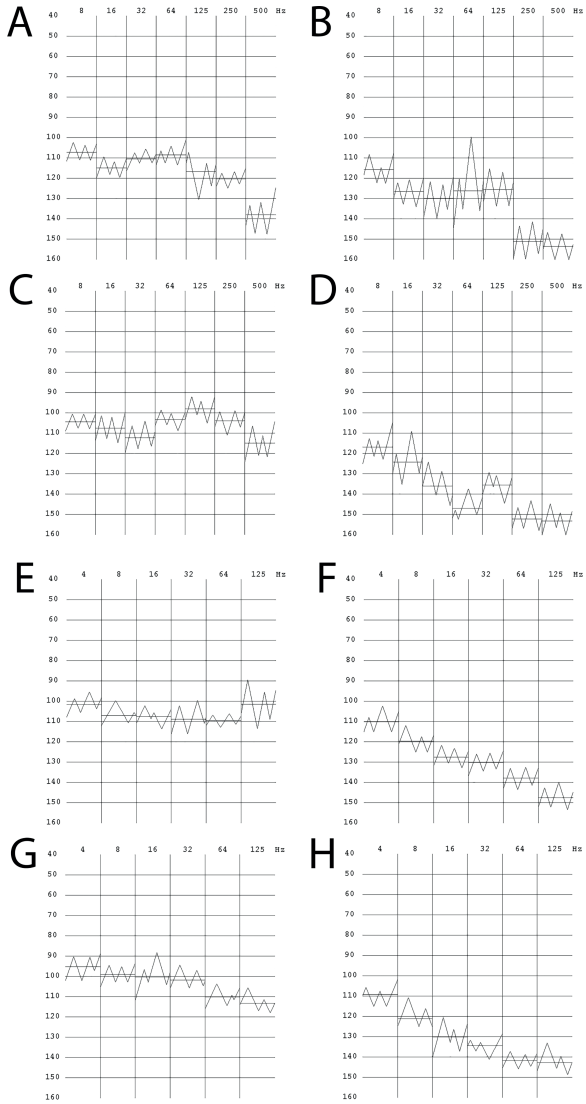

Fig 1. Vibrograms showing normal and abnormal VPTs. The vibrograms presented all arise from subjects with type 1 diabetes. (A) and (C) shows vibrograms reflecting normal vibrotactile sense, obtained from a 9-year old girl, at index and little fingers respectively. (B) and (D) shows vibrograms mirroring impaired vibrotactile sense, at index and little fingers respectively, in a 9-year old girl. (E) and (G) shows normal vibrograms, obtained from a 14-year old boy, at MTH 1 and MTH 5 respectively. (F) and (H) shows vibrograms, obtained from a 14-year old boy, mirroring impaired vibrotactile sense, at MTH 1 and MTH 5 respectively.

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Table 1. Characteristics of subjects.

Subjects Characteristics	All (n = 72) *	Boys (n = 39) †	Girls (n = 33) ‡	p-values §	Duration ≤ 5.3 years (n = 36) | |	Duration > 5.3 years (n = 36)	p-values ¶
Age	12.8 [11.5–15.0]	13.1 [10.9–14.8]	12.8 [11.7–15.2]	p = 0.848	12.0 [11.2–14.5]	14.0 [12.4–15.9]	**p = 0.011**
Age at onset	6.9 [4.7–10.3]	6.8 [4.8–10.8]	7.2 [4.3–10.1]	p = 0.861	9.3 [7.3–12.1]	4.9 [2.7–6.4]	**p < 0.001**
Duration of disease	5.3 [2.9–8.6]	5.4 [3.2–8.6]	5.3 [2.3–8.8]	p = 0.888	2.9 [1.3–3.9]	8.6 [7.0–10.2]	**p < 0.001**
BMI SD	0.51 [-0.17–1.29]	0.27 [-0.53–0.94]	1.00 [0.30–1.62]	**p = 0.005**	0.27 [-0.52–1.29]	0.75 [0.27–1.29]	p = 0.149
HbA1c, last value % (mmol/mol)	7.3 [6.7–7.8] (57 [50–62])	7.4 [6.7–7.9] (57 [50–63])	7.3 [6.8–7.6] (56 [51–60])	p = 0.991	7.0 [6.5–7.8] (54 [48–62])	7.4 [6.9–8.0] (57 [52–64])	p = 0.131
HbA1c, 1-year mean % (mmol/mol)	7.4 [6.9–7.8] (57 [52–62])	7.3 [6.9–7.7] 56 [51–61]	7.4 [7.0–7.9] (58 [53–63])	p = 0.462	7.2 [6.7–7.6] (56 [49–60])	7.5 [7.1–8.0] (58 [54–64])	**p = 0.023**
HbA1c, 2-year mean % (mmol/mol)	7.4 [6.9–7.8] (57 [52–62])	7.4 [6.9–7.7] 57.0 [52–61]	7.3 [7.1–7.9] (57 [54–63])	p = 0.614	7.1 [6.7–7.7] (54 [49–61])	7.5 [7.2–7.9] (58 [55–63])	**p = 0.019**
Insulin administration: Insulin pump Insulin pen	n = 45 n = 27	n = 26 n = 13	n = 19 n = 14		n = 18 n = 18	n = 27 n = 9	
Insulin–IU/24h	40.8 [25.7–56.4]	36.2 [23.8–51.3]	42.9 [29.4–63.6]	p = 0.229	34.8 [19.4–56.4]	46.1 [32.4–59.4]	p = 0.073
Insulin–IU/kg/24h	0.8 [0.6–1.0]	0.8 [0.6–1.0]	0.9 [0.6–1.0]	p = 0.523	0.8 [0.5–1.0]	0.9 [0.7–1.0]	p = 0.193

Values are expressed as medians [lower quartile–upper quartile]. HbA1c-values are given as % and due to IFCC standard in parenthesis (mmol/mol). Significant p-values at 0.05 level are in bold.

* n = 68 for "HbA1c, 1-year mean" and n = 63 for "HbA1c, 2-year mean".

† n = 38 for "HbA1c, 1-year mean" and n = 35 for "HbA1c 2-year mean".

‡ n = 30 for "HbA1c, 1-year mean" and n = 28 for "HbA1c, 2-year mean".

§ Comparison of characteristics between boys and girls using Mann-Whitney U-test.

| | n = 32 for "HbA1c, 1-year mean" and n = 27 for "HbA1c, 2-year mean".

¶ Comparison of characteristics between subjects with a disease duration of less than, and more than, 5.3 years using Mann-Whitney U-test.

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Vibration perception thresholds

Examples of vibrograms, obtained from the subjects with T1D, showing normal and impaired vibrotactile sense on all four sites examined are shown in Fig 1. All vibrograms presented graphically are age and gender matched, illustrating that children and adolescents with T1D can present with both normal and pathological vibrograms.

VPTs, presented as median z-scores, related to previously collected normative data (18), are shown in S1 (index finger), S2 (little finger) and S3 (MTH 1 and MTH 5 of the foot) Tables. Using Mann-Whitney U-tests with Bonferroni correction for multiple analyses (k = 24), on flagged significant values, showed no differences neither between boys and girls, nor between the subjects with a disease duration above or below median.

Elevated VPTs in relation to LTPs

Due to the criteria stated above in the methods section, a total of 13 out of 72 subjects (18%) presented with at least one pathological site on the foot, and a total of two subjects (3%) showed general elevations of VPTs on all four sites examined. Three out of 72 subjects (4%) had three pathological sites, and four out of 72 subjects (6%) had two pathological sites. A total

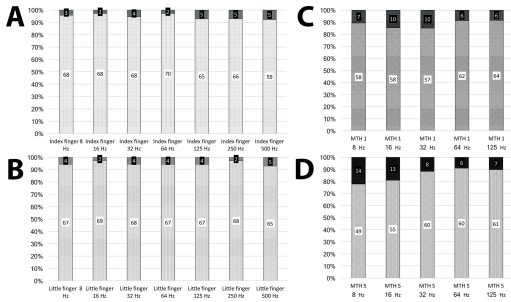

Fig 2. VPT graphs. These graphs show the number of subjects presenting with pathological (>1.96), and non-pathological (<1.96), z-scores at all examined frequencies and sites on the index **(A)** and little **(B)** fingers of the hand and on MTH 1 **(C)** and MTH 5 **(D)** on the foot. Z-scores are calculated based on normative values previously collected from healthy children and adolescents [21].

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of 64 out of the 72 subjects were also examined with Semmes-Weinstein's monofilaments. None of the subjects presented with impaired LTP at any of the sites examined.

Elevated VPTs in the foot and hand

From a group perspective, our data determined a higher occurrence of pathological frequencies, i.e. z-score >1.96, from the VPT examinations than would be expected comparing the obtained VPTs to our normative data. With a confidence interval of 95%, only 2.5% of the subjects should have VPTs with z-scores of >1.96 unless impaired vibrotactile sense is present in the group. Among the studied sites in the hand, the proportion of pathological VPTs ranged from 2.8 to 7.8%. In the foot, the proportion varied from 8.6 to 22.2%, with the highest proportion of pathological values presenting at low frequencies on both MTH 1 and MTH 5 (Fig 2).

Out of the 13 subjects presenting with impaired vibrotactile sense, three subjects had concurrent impaired vibrotactile sense in the hand. Only one subject presented with impaired vibrotactile sense in the hand, without having impaired sense in the foot at the same time.

Elevated VPTs in relation to treatment

Pathological VPTs on at least one site on the foot, seen in 13 out of 72 subjects, were more common among subjects treated with MDI (n = 9) compared to patients with CSII (n = 4) treatment (p = 0.013) (Fig 3F). No differences were seen comparing boys and girls (p = 0.760) (Fig 3E).

General characteristics for the subjects treated with MDI and CSII is presented in S4 Table. Children treated with MDI had older age at onset (p = 0.024), shorter disease duration (p = 0.025), higher daily dose of insulin (p = 0.015), but similar HbA1c levels, compared to those treated with CSII.

Elevated VPTs in relation to general characteristics

Median [quartiles] age, age at disease onset and disease duration of the subjects with at least one pathological site on the foot (n = 13) was 15.0 [11.5–16.8] years, 9.5 [5.7–12.6] years and 3.7 [1.8–7.1] years, respectively, and correspondingly among subjects with no pathological sites on the foot (n = 59) 12.7 [11.6–14.7] years, 6.8 [4.4–9.3] years and 5.5 [3.1–8.6] years, respectively. Age at disease onset was higher in the group of subjects presenting with at least

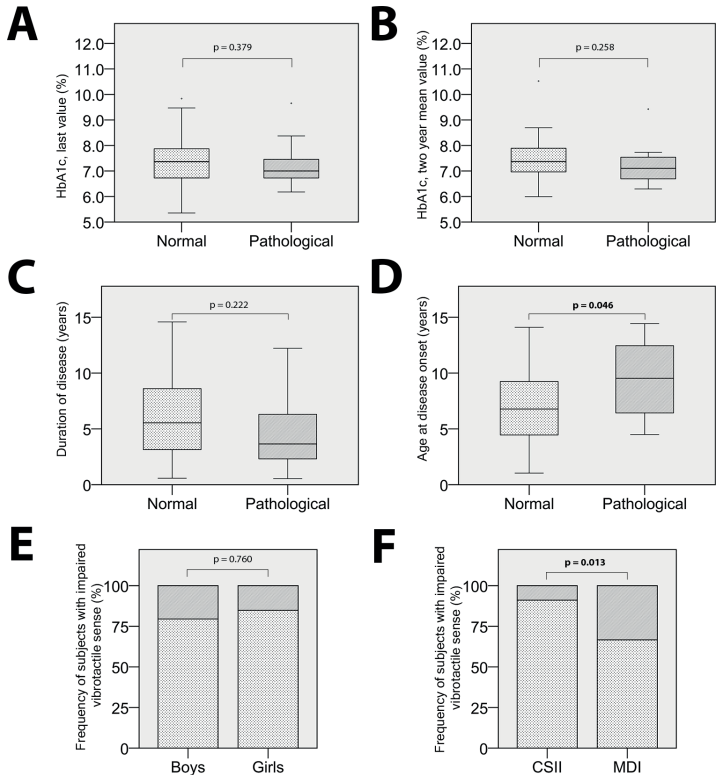

Fig 3. Boxplots and graphs. Neither last **(A)**, nor two-year mean **(B)**, HbA1c values differed between subjects with normal and impaired vibrotactile sense on at least one site on the foot. The duration of disease **(C)** did not statistically differ between subjects with normal and impaired vibrotactile sense on at least one site on the foot. However, among subjects with impaired vibrotactile sense, on at least one site of the foot, disease onset age was significantly higher **(D)**. The frequency of subjects with impaired vibrotactile sense, darker areas of the histograms, did not differ among boys and girls **(E)**, but subjects treated with MDI were more likely to have impaired vibrotactile sense **(F)**, than subjects treated with CSII. Among the 13 subjects with impaired vibrotactile sense four were receiving CSII treatment, and nine MDI treatment.

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one pathological site on the foot (p = 0.046) compared to the subjects with no pathological sites on the foot (Fig 3D). No such differences were seen comparing duration of disease (p = 0.222) (Fig 3C), last (p = 0.379) (Fig 3A) and two-year mean (p = 0.258) (Fig 3B) HbA1c, nor comparing age (p = 0.222), in groups with normal or abnormal VPTs. Furthermore, no differences were seen regarding normal or impaired vibrotactile sense in comparison with height (p = 0.263), weight (p = 0.353) or BMI SD (p = 0.356).

Screening for microalbuminuria in Sweden is regularly started at the age of 10. In 61 out of the 72 subjects, data on microalbuminuria was available. None of these 61 subjects, including all subjects with altered vibrotactile sense, showed signs of microalbuminuria at the time of vibrotactile examination. Blood pressure, available in 60 participants, were all within normal

limits. Likewise, 67 out of 72 subjects, including all subjects with altered vibrotactile sense, had data on the presence of retinopathy in their medical records. Five subjects (7%) showed signs of mild retinopathy at the time of the vibrotactile examination, and two out of these five subjects showed concurrent impaired vibrotactile sense. Fishers exact test showed that retinopathy was not more common among subjects with neuropathy, and vice versa ($p = 0.247$).

Discussion

In this study, we were, to our knowledge, for the first time investigating vibrotactile sense in children and adolescents with T1D, using multi-frequency vibrometry. A total of 13 out of 72 (18%) subjects presented with impaired vibrotactile sense on at least one site on the foot, compared to age and gender matched controls, showing us that it is possible to identify children and adolescents with signs of underlying DPN using multi-frequency vibrometry.

Our data clearly indicate that subjects treated with MDI were more likely to show signs of impaired vibrotactile sense than those treated with CSII, although they had similar quality of glycaemic control and shorter diabetes duration. This is similar to what was presented by Zabeen et al., showing that treatment with CSII is connected to lower rates of peripheral nerve abnormalities [24]. A possible explanation to this is that CSII is associated with a more even distribution of insulin than MDI that might not be reflected in HbA1c values. Another study has shown that CSII, compared to MDI, treatment was associated with regeneration of the corneal nerve fibers, although HbA1c values did not differ between the groups [25]. This is interesting and suggests that CSII treatment is somewhat connected to a better survival and growth environment for the nerve fibers. It is well established that increased metabolic control can decelerate the development of DPN in an adult population of subjects with T1D [26]. Furthermore, the most significant risk factor of developing DPN is poor metabolic control, reflected by increased HbA1c values [27]. In our study, we did not find any correlation between higher HbA1c values and the presence of elevated VPTs. A possible explanation might be that the subjects of our study are, in comparison to previous studies, presenting with lower HbA1c values. Additionally, since HbA1c reflects an average of the blood glucose level over time, short-timed spikes of high and low blood glucose levels may not be reflected. Our results support the theory that glycemic variability might play a role in DPN development in subjects with T1D [28–30]. However, in order to establish this correlation, it would be necessary to follow the subjects over time with at least two VPT examinations, with a substantially long follow up period, as well as equipping the subjects with continuous glucose monitors.

Furthermore, our data suggests that an older age at onset of T1D might somehow be connected to impaired vibrotactile sense on the foot. This might be explained by the better plasticity of peripheral nerves in younger children, compared to adolescents, as well as the brains ability to adapt to changes after nerve damage, as previously reported in children and adolescents undergoing nerve repair due to a median nerve injury [31, 32]. Therefore, screening for subclinical DPN in children and adolescents with T1D is important to enable an early detection and establishment of optimized glycemic control. Similarly, diabetic nephropathy, another well-known microangiopathic complication to T1D, has been shown to be more common among subjects with a later disease onset [33, 34].

Retinopathy is another well-known complication to T1D, and therefore children and adolescents are screened for this during child- and adulthood [15]. Previous studies have suggested that neuropathy, measured with corneal confocal microscopy, may precede both retinopathy and microalbuminuria in adults with T1D, and that small nerve fiber dysfunction may precede large nerve fiber dysfunction [35, 36]. As presented earlier, data on retinopathy was present in 67/72 subjects of this study. Only five subjects had retinopathy (all mild), and

two out of these five subjects showed concurrent impaired vibrotactile sense, concluding that neuropathy, present in 13 out of 72 subjects, seem to be more common than retinopathy in our study.

In contrast to most studies, we chose to measure the VPTs and LTP on finger pulps and foot soles because these sites are tactile surfaces. Therefore, it is likely to believe that they better correlate to actions performed by the hands and feet than non-tactile surfaces. The presently used sites are different from the ones used in the reviewed articles by Hirschfeld et al., where VPTs were solely obtained from the feet, and not from hands [10]. In our study, we have shown that impaired vibrotactile sense is more common in the foot than in the hand, which supports the theory that the nerves in the lower limbs are affected by DPN before the nerves in the upper limbs, due to the length of the nerves [14]. Previous studies have also shown that nerve conduction amplitudes decrease with the height of the subject, but in our study we did not see any correlation between height and impaired vibrotactile sense [37].

A recent position statement for diabetic neuropathy, claims that a 128 Hz tuning fork can be used for assessment of vibration perception [13]. In Sweden, a 128 Hz tuning fork is recommended when screening for peripheral neuropathy among patients with diabetes, but this method has got a low sensitivity in the detection of DPN [13, 38, 39]. Since more pathological VPTs are present at 16 Hz than at 125 Hz, on both sites examined on the foot, questions are raised on the eligibility of using a 128 Hz tuning fork in regular screening for DPN.

The most obvious limitation to this study is that the subjects have not been examined with an electrophysiology method, measuring nerve conduction velocities and amplitudes. Although 13/72 subjects (18%) were identified with at least one pathological examined site on the foot, comparisons must be made between the VibroSense Meter and electrophysiology in order to establish the sensitivity of the method. However, the results of the measurements in the patients with T1D were compared to normal data from 269 school children aged eight to 20, and the finding should therefore be reliable. Another limitation is that the children and adolescents have not undergone a clinical neurological examination or examinations with regular screening tools, apart from monofilaments, such as a biothesiometer and tuning forks. Such examinations have been made by Blankenburg et al. (2012), and according to their findings tactile detection, using von Frey filaments, was a better screening tool for DPN than vibration testing, using a Rydel-Seifer tuning fork [11]. None of the children or adolescents in our study had any clinical signs of neuropathy, such as numbness or pain in palms or foot soles, according to their medical records. A possible weakness is that the subjects have only undergone one examination of their vibrotactile sense, but a previous study using the Vibrosense Meter has emphasized that there is a strong reliability of the technique in the test-retest of patients with neuropathy; in that case hand arm vibration syndrome (i.e. HAVS) [40].

The lack of data regarding Tanner stage of the subjects is a limitation that could possibly explain why subjects with older disease onset age, and thereby more likely to have entered puberty, present with a higher proportion of impaired vibrotactile sense. A similar correlation has been shown by Barkai et al., showing that puberty is a risk factor for diabetic neuropathy [41]. However, the subjects z-scores were compared with an age and gender matched healthy population of children and adolescents, where no Tanner stages were judged [21].

Conclusions

Signs of DPN, not identified using Semmes-Weinstein's monofilaments, can be detected in children and adolescents with T1D by the Vibrosense Meter. Since as many as 18% of the subjects had signs of DPN, screening is important. Further studies are needed to validate the

finding of the increased risk of DPN with older age at onset and treatment with MDI compared to CSII.

Supporting information

S1 Fig. Example of visibly incorrect vibrograms. An example of a visibly incorrect frequency is shown in **(A)** at 500 Hz. Prior to statistical analysis the VPT value of 500 Hz is being excluded. **(B)** is showing a vibrogram being excluded in whole, due to the pattern of the curves, as well as the lack of VPT at 8 Hz. Even 4 and 250 Hz seem to be lacking in **(B)**, but these frequencies have not been examined used in the present examination.
(TIF)

S1 Table. Z-scores of VPTs obtained from index finger. Median [lower quartile–upper quartile] values of z-scores from VPTs at all frequencies obtained from index finger on the right hand. Comparisons, using Mann Whitney U-tests, are made between boys and girls, and between subjects with a disease duration of less than and more than 5.3 years. P-values are presented and significant p-values, at 0.05 level, are corrected with Bonferroni corrections for multiple analyses (k = 24) and presented in parenthesis.
(DOCX)

S2 Table. Z-scores of VPTs obtained from little finger. Median [lower quartile–upper quartile] values of z-scores from VPTs at all frequencies obtained from little finger on the right hand. Comparisons, using Mann Whitney U-tests, are made between boys and girls, and between subjects with a disease duration of less than and more than 5.3 years. P-values are presented and significant p-values, at 0.05 level, are corrected with Bonferroni corrections for multiple analyses (k = 24) and presented in parenthesis.
(DOCX)

S3 Table. Z-scores of VPTs obtained from metatarsal heads one and five on the foot. Median [lower quartile–upper quartile] values of z-scores from VPTs at all frequencies obtained from MTH 1 and MTH 5 on the right foot. Comparisons, using Mann Whitney U-tests, are made between boys and girls, and between subjects with a disease duration of less than and more than 5.3 years. P-values are presented and significant p-values, at 0.05 level, are corrected with Bonferroni corrections for multiple analyses (k = 24) and presented in parenthesis.
(DOCX)

S4 Table. Characteristics of subjects divided by treatment methods. Values are expressed as medians [lower quartile–upper quartile]. HbA1c-values are given as % and due to IFCC standard in parenthesis (mmol/mol). Significant p-values at 0.05 level are in bold.
* n = 42 for "HbA1c, 2-year mean".
† n = 38 for "HbA1c, 1-year mean" and n = 35 for "HbA1c 2-year mean".
‡ n = 23 for "HbA1c, 1-year mean" and n = 21 for "HbA1c, 2-year mean".
§ Comparison of characteristics between subjects treated CSII and subjects treated with MDI using Mann-Whitney U-test.
(DOCX)

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Paper II

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BRIEF REPORT

ACTA PÆDIATRICA NURTURING THE CHILD WILEY

Impaired vibrotactile sense showed no association with insulinoma associated protein 2 and glutamic acid decarboxylase autoantibodies in paediatric type 1 diabetes

Diabetic peripheral neuropathy (DPN) is a well-known complication of type 1 diabetes in children and adults and may present early after diagnosis. It is frequently asymptomatic in young patients, but can be confirmed using screening tools, such as monofilaments, tuning forks or a biothesiometer. Electrophysiology is the gold standard examination for detecting DPN, but is seldom used due to lack of resources and because it is time-consuming, expensive and possibly unpleasant for subjects.[1] Multi-frequency vibrometry can also determine impaired vibrotactile sense, a sign of large nerve fibre pathology.[2] We previously studied this tool in 72 asymptomatic children and adolescents aged 8-18 with type 1 diabetes and 13 had impaired vibrotactile sense.[2] Louraki et al[3] suggested that sensory nerve action potentials reflecting amplitudes were lower among diabetic subjects with autoantibodies against insulinoma associated protein 2 (IA-2) and glutamic acid decarboxylase (GAD65). These lower levels indicated early axonal dysfunction and implied a possible connection between DPN and autoimmunity in type 1 diabetes.[3] However, no correlations were seen between IA-2 and GAD65 positivity in a pathological nerve conduction study.[3]

We previously studied the current cohort with regard to potential relationships between impaired vibrotactile sense and common associated risk factors for DPN in type 1 diabetes. These included age, gender, disease duration, body mass index and glycated haemoglobin (HbA1c).[2] We could not detect any association, but subjects were more prone to impaired vibrotactile sense if they had multiple daily insulin injections than insulin pumps.[2]

Our aim was to investigate potential associations between impaired vibrotactile sense in paediatric type 1 diabetes and the presence of IA-2 and, or, GAD65 autoantibodies.

Vibrotactile sense was examined using a VibroSense Meter version 1 (VibroSense Dynamics, Malmo, Sweden) and compared to previously described normative data.[2] Data on autoantibodies to IA-2 and GAD65 were collected at the time of the type 1 diabetes diagnosis. The subjects were consecutively recruited from the Swedish paediatric outpatient clinics at Skåne University Hospitals in Malmö and Lund, and Helsingborg Hospital, between April 2015 and June 2016. All patients aged 8-18 who were diagnosed with type 1 diabetes were asked to participate. The inclusion and exclusion criteria have previously been described.[2] We included all type 1 diabetes patients aged 8-18 interested in participating and excluded patients with other diseases known to present with impaired vibrotactile sense. Patients with concurrent coeliac and, or, autoimmune thyroiditis were not excluded.[2]

Data on autoantibodies were collected after inclusion.[2] All the statistical analyses were carried out with Fisher's exact test, using SPSS Statistics, version 25 (IBM Corp, New York, USA). Informed assent and consent were obtained from all the subjects and their parents or their legal guardians. The research was conducted in accordance with the Declaration of Helsinki. The local ethics committee at Lund University, Sweden, approved the study (386/2007).

We previously found that 13/72 (18%) subjects showed impaired vibrotactile sense.[2] Their median (quartiles) age was 12.8 (11.5-15.0) years, disease duration was 5.3 (2.9-8.6) years, and their age at disease onset was 6.9 (4.7-10.3) years. There were data on autoantibodies at diagnosis for 70/72 (97%) subjects, including 12/13 (92%) with impaired vibrotactile sense. Of these, 58 (83%) had positive IA-2 autoantibodies, 45 (64%) were GAD65 positive, 39 (56%) were positive for both, and six (9%) were antibody negative. Fisher's exact test showed no association between impaired vibrotactile sense and autoantibodies to IA-2 ($P = 1.0$), GAD65 ($P = .74$) or both ($P = 1.0$).

The pathogenesis of DPN in type 1 diabetes is not fully understood, but several processes have been suggested. Autoimmunity may play a role in the development of type 1 diabetes and complications like DPN. However, conflicting results have been reported, for example for GAD-autoantibody positivity and impaired nerve function.[3,4]

We did not find any associations between impaired vibrotactile sense and IA-2 or GAD65 autoantibodies after an average disease duration of 5.3 years. The autoantibody levels may have been too low to impact the nerves, but the relatively small number of subjects must also be considered. We do not know the type or level of autoantibodies present when vibrotactile sense was examined. This makes it impossible to draw further conclusions from the possible effect of high levels of autoantibodies on the peripheral nervous tissue. Our results agreed with Hoeldtke et al's[5] findings that GAD65 autoantibodies did not increase among patients with neuropathy.

Lars B. Dahlin and Helena Elding Larsson share senior authorship.

In conclusion, our data do not support an association between DPN and GAD65 autoantibodies or between DPN and IA-2 autoantibodies.

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CONFLICTS OF INTEREST

None.

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Paper III

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REGULAR ARTICLE

ACTA PÆDIATRICA NURTURING THE CHILD WILEY

Vibrotactile sense might improve over time in paediatric subjects with type 1 diabetes—A mid-term follow-up using multifrequency vibrometry

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Abstract

Aim: Impaired vibrotactile sense, mirroring diabetic peripheral neuropathy, is present among children and adolescents with type 1 diabetes. This study aims to re-examine the vibrotactile sense of paediatric type 1 diabetes subjects in order to evaluate any alterations in the vibrotactile sense over time.

Methods: A VibroSense Meter I device was used to determine the vibrotactile perception thresholds (VPTs) for seven frequencies from the pulp of index and little fingers and for five frequencies from metatarsal heads one and five on the sole of the foot, of 37 children and adolescents with type 1 diabetes, previously examined in a larger cohort. Subjects were followed up after a median time of 30 months. Z-scores of VPTs were calculated using previously collected normative data.

Results: Vibrotactile perception thresholds improved over time at low frequencies (especially 16 Hz) on the foot, while not being statistically significant different on the rest of the frequencies, either on hand or foot. VPTs were not correlated with HbA1c.

Conclusion: A mid-term follow-up of vibrotactile sense in paediatric subjects with type 1 diabetes shows a conceivable normalization of previously impaired vibrotactile sense on some frequencies on the foot, indicating that vibrotactile sense might fluctuate over time.

KEYWORDS
neuropathy, type 1 diabetes, vibration perception threshold, vibrotactile sense

1 | INTRODUCTION

Children and adolescents with type 1 diabetes (T1D) are at risk of developing microvascular complications to their disease at an early stage of the disease and at young age.[1,2] The most common complications are diabetic retinopathy and diabetic nephropathy, but also diabetic neuropathy is present among paediatric T1D subjects.[3–5] ISPAD Clinical Practice Consensus Guidelines 2018 state that screening for microvascular complications should be commenced, starting at the age of "11 years with 2-to-5-year diabetes duration and annually thereafter".[1] The American Diabetes Association's paediatric guidelines ("Standards of Medical Care in Diabetes—2021"),

Abbreviations: DPN, diabetic peripheral neuropathy; MTH 1, metatarsal head 1; MTH 5, metatarsal head 5; T1D, type 1 diabetes; VPT, vibration perception threshold.

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regarding screening for neuropathy, are similar; "consider an annual comprehensive foot examination at the start of puberty or at age ≥10 years, whichever is earlier, once the youth has had type 1 diabetes for 5 years".[2]

Screening for neuropathy should include screening for small- as well as large-fibre pathology, but the gold standard method for detecting diabetic peripheral neuropathy (DPN) is nerve conduction studies, measuring conduction velocities in large nerve fibres in response to sensory and/or motor stimuli.[3] Screening for large-fibre pathology is in outpatient clinics mostly performed on children and adolescents, as well as on adults, using a single 10-g monofilament and/or a tuning fork for detection of impaired tactile and vibrotactile sense, respectively.[2,3] These screening methods have shown to be easy to perform, but lack in sensitivity and specificity, especially in paediatric patients.[5,6]

With all screening tools, it is important to be able to repeatedly measure the sense in the same manner time after time, in order to compare the results with each other and to detect alterations in tactile and/or vibrotactile sense. The reproducibility of vibration perception thresholds (VPTs), obtained using a biothesiometer, has been studied in paediatric T1D subjects, showing that T1D subjects had higher testing variability compared to controls, suggesting a better accuracy when testing healthy controls compared to subjects with impaired nerve function.[7] In the aforementioned study, the reproducibility was tested at two times on the same date, potentially leading to short-term learning effects influencing the results.[7]

We have previously shown that impaired vibrotactile sense, presented as elevated VPTs when subjects are examined using the Vibrosense Meter, is present among children and adolescents with T1D, suggesting that DPN occurs in children and adolescents with T1D.[8] Our previous results indicated no association between impaired vibrotactile sense and the presence of T1D-associated autoantibodies,[9] in contrast with findings by Louraki et al., suggesting lower sensory nerve action potentials among subjects with autoantibodies to insulinoma-associated protein 2 and glutamic acid decarboxylase.[10] A study on adult T1D subjects has also suggested that an improved metabolic control could lead to improvement in VPTs, raising questions on whether nerve dysfunction in T1D to some extent might be reversible.[11] Two-week interval test–retest reliability has been tested for the Vibrosense Meter, with intra-class correlation coefficients ranging from 0.59 to 0.93, depending on site, among vibration exposed workers.[12] Furthermore, practice effect for use of the Vibrosense Meter has been tested longitudinally on healthy individuals, resulting in a relatively small effect of continuous training, especially with an increased testing interval.[13]

The aim of this study was to re-examine children and adolescents with T1D, already examined with the Vibrosense Meter [data previously published[8]], in order to study the vibrotactile sense over time using the Vibrosense Meter and to study if fluctuations in VPTs might occur in relation to altered HbA1c levels or other characteristics.

Key Notes

- Diabetic peripheral neuropathy is present in children and adolescents with type 1 diabetes
- Diabetic peripheral neuropathy is thought to be dependent on increasing duration of type 1 diabetes and/or inadequate metabolic control
- A mid-term follow-up of vibrotactile sense among subjects with type 1 diabetes indicates that vibrotactile sense might fluctuate over time although HbA1c might deteriorate

2 | SUBJECTS AND METHODS

2.1 | Subjects

In the previous study, 72 children and adolescents (boys = 39) with T1D were included for vibrotactile examinations and statistical analysis. The participants were recruited from the paediatric outpatient clinics at Skåne University Hospital and the Hospital of Helsingborg. In the current study, all 72 patients from the previous study were asked to participate in a follow-up study. Data from the previous study, i.e., baseline data, were collected between June 2015 and April 2016, and the follow-up data were collected between April 2018 and August 2018. A clinical neurological examination was not carried out, but a thorough review of the medical records was done, both at baseline and at follow-up, in order to find signs of clinical neuropathies.[8]

2.2 | Vibration perception thresholds

Vibrotactile perception thresholds were obtained using a standard, as well as a modified, VibroSense Meter I device for measurements of the right hand and foot, respectively. On the right hand, vibrotactile sense was measured at seven frequencies (8, 16, 32, 64, 125, 250 and 500 Hz) at the pulp of index and little fingers, reflecting the median and ulnar nerves, respectively. On the right foot, measurements were obtained from the sole of the foot at five frequencies (8, 16, 32, 64 and 125 Hz) at metatarsal heads one (MTH 1) and five (MTH 5), reflecting the function of the medial and lateral branches of the tibial nerve, respectively. The subjects, wearing hearing protectors and placed in a secluded room to minimize disturbance, were to press a remote control when perceiving vibrations on the measurement sites.[14] For technical details on determination of each specific VPT at each frequency, please see Dahlin et al 2015.[14] This results in numerical values, as well as curves with thresholds describing the vibrotactile sense, i.e. VPTs.

Z-scores of VPTs, for all frequencies and sites tested, were calculated in comparison with previously collected normative data of age- and gender-matched controls.[14] As previously described, all VPT curves, i.e., vibrograms, were manually and visibly scrutinized and apparently incorrect VPTs, reflecting lack of concentration or

inability to comply with the examination, were excluded from statistical analysis.[8] An example of a visibly incorrect VPT, due to insufficient curve endpoints, and/or an examination that is completely incorrect, due to lack of interest or compliance is presented in Figure S1. Mean of VPT z-scores from every site was calculated from the first examination and at follow-up and compared as described below.

A more thorough description of the examination using the Vibrosense Meter I can be found in the previous study by Ising et al.[8] and in an article by Dahlin et al. describing the method as well as the used normative data from healthy children and adolescents.[14]

2.3 | Perception to light touch

A 20-piece Semmes–Weinstein's monofilament collection [Touch-TestTM, North Coast Medical Inc.; filaments ranging from 1.65 (0.008 g) to 6.65 (300 g)] was used to determine light touch perception (LTP). LTP was obtained by applying the monofilaments, starting with the thinnest and advancing to thicker monofilaments if not perceived, at the same sites as VPTs were obtained. Normal tactile sensitivity in the hand and foot was defined as being able to perceive tactile stimulus to monofilaments ≤2.83 (0.07-g) and ≤3.61 (0.4-g), respectively, as described earlier and in compliance with the manufacturer's guidelines.[8]

2.4 | Statistical analyses

Baseline and follow-up characteristics are presented as medians [quartiles] for the entire study group, and for boys and girls, respectively. Differences in characteristics were tested using Mann–Whitney U-tests.

Differences in VPTs between the first examination and follow-up, for all sites and frequencies tested, were analysed using Wilcoxon signed rank test. Bonferroni corrections were performed on statistically significant values, i.e., $p < 0.05$, due to multiple testing of VPTs from all frequencies and sites tested ($n = 24$). Furthermore, in order to make an entire examination, and not only single frequencies, comparable between the first examination and follow-up, the mean of VPT z-scores from every site was calculated and compared, using Wilcoxon signed rank tests. Correlations between mean VPT z-scores from all sites and HbA1c at follow-up were calculated using Spearman's correlations.

All statistical analyses were made using IBM SPSS Statistics version 27 for Mac.

2.5 | Ethics

The local ethics committee at Lund University approved the study (386/2007). Written informed consents were obtained from all subjects as well as their legal guardians. All research is conducted in accordance with the Declaration of Helsinki.

3 | RESULTS

3.1 | Subjects

A total of 37, out of originally 72 (boys = 39), children and adolescents (boys = 20) accepted the invitation for follow-up. Characteristics of the subjects, presented for all subjects as well as for girls and boys separately, from baseline and follow-up are presented in Table 1. Median [quartiles] follow-up time was 30 [25–32] months, with no difference between boys and girl ($p = 0.99$).

Data from the 35 children and adolescents that did not choose to participate in the follow-up study, i.e., non-attenders, are solely available from the baseline study. Age, age at onset of T1D, duration of T1D, BMI and the 2 year mean of HbA1c did not differ between the attending and not attending groups at follow-up. However, the 37 children and adolescents participating in the follow-up study had slightly lower HbA1c values at the first examination compared to the 35 children and adolescents not participating in the follow-up; median [quartiles] HbA1c value was 54 [50–58] among the 37 children participating in the follow-up compared to 59 [51–67] among the 35 children not participating ($p = 0.024$).

Median [quartiles] age and disease duration were 12.7 [11.5–14.8] and 5.3 [2.5–8.5] years at baseline and at follow-up 15.2 [13.6–17.3] and 7.9 [5.2–8.5] years, respectively. Median age for T1D onset was 6.8 [4.2–10.8] years. HbA1c levels were in median 54 [50–58] mmol/mol (7.1 [6.7–7.5] %) and 53 [50–65] mmol/mol (7 [6.6–8.1] %) at baseline and follow-up, respectively ($p = 0.21$), and the median change from baseline to follow-up was 0 [−7–10] mmol/mol (0 [−0.6–0.9] %). Change in HbA1c differed significantly between boys and girls; −4 [−7–6] mmol/mol (−0.4 [−0.6–0.5] %) vs. 7 [−2–18] mmol/mol (0.6 [−0.2–1.7] %) ($p = 0.015$), respectively.

3.2 | Vibration perception thresholds

Median z-scores for each frequency and site for all subjects, at baseline and follow-up, as well as the median z-score difference, for hand and foot are presented in Tables S1 and S2, respectively. Z-scores differed significantly on group level at 16 Hz on MTH 1 and MTH 5 (statistical significance remained after Bonferroni correction). Z-scores of VPTs at 16 Hz on MTH 1 (corrected p-value = 0.024) and MTH 5 (corrected p-value = 0.024) were significantly higher at baseline than at follow-up, indicating improvement or normalization of vibrotactile sense. Median difference of z-scores was not associated, at any frequency or site, with either sex or HbA1c being stable/decreased or increased (Tables S1 and S2).

Moreover, raw scores of VPTs from baseline and follow-up at all frequencies and sites are presented as median [quartiles] across all samples in Tables S3 and S4. VPTs were, across all samples, significantly lower at 250 Hz on both index (corrected p-value < 0.024) and little fingers (corrected p-value = 0.024) at follow-up compared to baseline. On the foot, VPTs were, across all samples, significantly

TABLE 1 Baseline and follow-up characteristics

		All ($n = 37$)	Boys ($n = 20$)	Girls ($n = 17$)	Boys vs Girls (p-value) *	Stabile/decreased HbA1c ($n = 19$)	Increased HbA1c ($n = 18$)	Stabile/decreased vs increased HbA1c*
Age	Baseline	12.7 [11.5–14.8]	12.9 [11.0–14.7]	12.5 [11.7–15.0]	0.81	12.7 [11.5–14.2]	12.9 [11.4–15.2]	0.78
	Follow-up	15.2 [13.6–17.3]	15.3 [13.1–17.1]	15.2 [13.8–17.6]	0.76	15.2 [14.0–16.7]	15.3 [13.5–17.9]	0.86
Onset age		6.8 [4.2–10.8]	6.6 [4.5–10.3]	7.2 [2.7–11.6]	0.90	5.9 [2.9–7.5]	8.8 [4.7–12.5]	0.052
Duration, years	Baseline	5.3 [2.5–8.5]	5.3 [2.9–8.6]	5.3 [1.9–8.7]	0.78	5.7 [4.1–9.1]	3.5 [0.7–5.9]	**0.027** *
	Follow-up	7.9 [5.2–10.8]	7.9 [5.5–10.8]	7.9 [4.3–10.9]	0.90	8.3 [6.2–11.8]	6.1 [3.3–8.3]	**0.030** *
HbA1c mmol/mol (%)	Baseline	54 [50–58] (7.1 [6.7–7.5])	57 [49–63] (7.4 [6.7–7.9])	52 [50–55] (6.9 [6.7–7.2])	0.08	55 [50–62] (7.2 [6.7–7.8])	53 [48–57] (7.0 [6.6–7.4])	0.20
	Follow-up	53 [50–65] (7 [6.6–8.1])	53 [50–64] (7.0 [6.7–8.0])	57 [49–68] (7.4 [6.6–8.4])	0.40	51 [46–53] (6.8 [6.4–7.0])	64 [57–71] (8.0 [7.4–8.7])	**<0.001** ***
Change in HbA1c mmol/mol (%)		0 [−7–10] (0 [−0.6–0.9])	−4 [−7–6] (−0.4 [−0.6–0.6])	7 [−2–18] (0.6 [−0.2–1.7])	**0.015** *	−6 [−7 – −3]	10 [7–21]	**<0.001** ***
Follow-up time, months		30 [25–32]	30 [25–32]	31 [25–32]	0.99	31 [26–32]	28 [25–32]	0.31

Note: Values are expressed as medians [quartiles]. HbA1c is given as IFCC standard and as % in parenthesis. p-values are considered significant at the 0.05 level.

*Comparison between characteristics between boys and girls, as well as between subjects with stabile/decreased and increased HbA1c, using Mann–Whitney U-test; $p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

lower at 16 Hz on first metatarsal head of the foot (corrected *p*-value = 0.024) at follow-up compared to baseline. At no frequency or site were VPTs significantly higher at follow-up compared to baseline.

In order to compare an entire site between two examinations, mean VPT z-scores are presented for the entire sites examined (i.e. index finger, little finger, MTH 1 and MTH 5), and comparisons are made using Wilcoxon signed rank tests with $p < 0.05$ considered as statistically significant. Boxplots for VPT z-scores at baseline and follow-up are presented in Figure 1. Mean z-scores (±SD) at baseline vs follow-up were −0.091 (±1.251) versus −0.227 (±1.110) for index finger and −0.119 (±1.228) versus −0.432 (±1.100) for little finger. Using Wilcoxon signed rank tests showed no difference between z-scores at baseline versus follow-up at index ($p = 0.227$) or little fingers ($p = 0.058$). At MTH 1, z-scores were significantly higher at baseline versus follow-up (0.438 (±1.401) versus −0.137 (±1.116); $p = 0.016$), while z-scores at MTH 5 did not differ significantly between baseline vs follow-up (0.558 (±1.460) versus 0.107 (±1.343); $p = 0.062$).

Mean VPT z-scores did not correlate with HbA1c at follow-up at any site. Correlation coefficients (*p*-values) were 0.047 ($p = 0.785$) for index finger, 0.152 ($p = 0.370$) for little finger, −0.151 ($p = 0.373$) for MTH 1 and −0.162 ($p = 0.337$) for MTH 5.

3.3 | Perception to light touch

At baseline, no subjects had any abnormalities in the perception to light touch (data previously published).[8] At follow-up, four out of 37 subjects (boys = 2) had at least one site with impaired LTP regarding to the manufacturers recommended level of LTP thresholds for children and adolescents. On group level, LTP thresholds were significantly higher at follow-up on both index and little fingers ($p = 0.006$ and $p = 0.002$, respectively), while no significant differences were seen on MTH 1 and MTH 5 at follow-up compared to baseline ($p = 0.40$ and $p = 0.46$, respectively).

4 | DISCUSSION

In this study, we present follow-up data on vibrotactile sense, obtained with a VibroSense Meter, on children and adolescents with T1D, after a median follow-up time of 30 [25–32] months. The original baseline study, previously published by Ising et al., included a total of 72 children and adolescents, and 37 of them have been re-examined in the present follow-up study,[8] where the attending 37 children and adolescents only differed, at baseline, with slightly lower HbA1c values compared to the 35 non-attending children and adolescents.

Evaluation of all examined frequencies and sites showed no statistically significant difference in z-scores between baseline and follow-up, except for 16 Hz on MTH 1 and MTH 5, where VPTs were higher at baseline compared to follow-up. Moreover, median VPT raw scores were lower across all samples at follow-up compared to baseline at 250 Hz on both sites on the hand and on 16 Hz on MTH1. Normalization of VPTs, although the subjects age and disease duration increase, raises a lot of questions regarding the vibrotactile sense and whether it can improve over time, or if there are natural fluctuations in the vibrotactile sense. Possible explanations might be that both the peripheral and the central nervous systems have the ability to regenerate any degenerated nerve fibres, as well as compensate any dysfunction with peripheral and central plasticity, respectively, especially in paediatric subjects. However, it is plausible that VPTs can increase or decrease over time in relation to age as has been shown in healthy subjects.[14] Thus, we suggest using z-scores, instead of raw data, when looking at VPTs in subjects with T1D, since individual data are then adjusted in relation to age- and gender-matched controls.[14]

No abnormalities were found to LTP at baseline. However, at follow-up, four out of 37 subjects presented with impaired LTP, defined as inadequate tactile response to a monofilament examination based on the manufacturer's guidelines, i.e., ≤0.07-g monofilament on the hand and ≤0.4-g monofilament on the foot. These thresholds are, nonetheless, much lower than the recommended screening

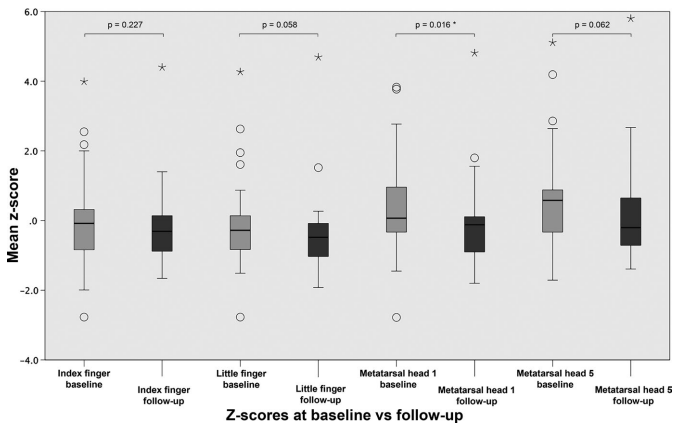

FIGURE 1 Mean z-scores, across all samples, for all frequencies tested at index finger, little finger, metatarsal head 1 and metatarsal head 5 are calculated and presented from baseline and follow-up using boxplots. Comparisons of mean z-scores were made using Wilcoxon signed rank tests, and *p* values < 0.05 were considered statistically significant. *N* = 37

guidelines from the American Diabetes Association, which state that screening for DPN in children and adolescents, as well as for adults, should be done using a 10-g monofilament.[2,15] One may suggest that the use of thinner monofilaments should be considered and validated in the future with a possibility to find dysfunction based on DPN earlier.

We believe that the normalization of VPTs and concurrent impairment of LTPs are most likely a coincidence, rather than significant, and due to the small number of subjects in this study, it is impossible to draw certain conclusions from this relation. However, it is plausible that the sensation to light touch might react differently than the sensation to vibrations, since the tactile sense mostly depends on Merkel's discs and Meissner's corpuscles, whereas the vibrotactile sense mostly depends on Meissner's and Pacinian corpuscles.[16–18] Thus, no extensive conclusions can be drawn, neither from the deterioration of the LTPs, nor the possible normalization of VPTs.

Donaghue et al. reported a higher rate of peripheral nerve abnormalities at baseline investigations in newly diagnosed adolescent T1D subjects, compared to a follow-up, in median three years later.[19] Additionally, they found that abnormalities were not related to age, diabetes duration or glycaemic control.[19] In addition, Solders et al. have reported low sensory nerve conduction to be present at onset of T1D in paediatric subjects, and that it might improve over the first two years and then deteriorate again.[20] This shows that nerve function might improve, which is in accordance with the present study. However, the subjects in the present study have a longer disease duration than the ones in the study by Solders et al.[20] On the other hand, Lee et al. reported in a prospective 5-year follow-up study on nerve conduction from newly diagnosed adolescent T1D subjects that 32% showed evidence of peripheral neuropathy in at least two nerves at time of diagnosis and that nerve conduction abnormalities rose during the 5-year follow-up, except for variables in the sural nerve.[21] Furthermore, Lee et al. reported that the parameters affecting nerve conduction the most were diabetes duration and sustained hyperglycaemia.[21] In the present study, HbA1c was significantly higher among girls than among boys at follow-up compared to baseline. However, both girls and boys presented with fewer pathological VPT examinations at follow-up than at baseline. A possible explanation might be that the hyperglycaemia has to be sustained for a longer period to influence and impair the vibrotactile sense. The above-mentioned reports suggest that DPN in T1D is multifactorial and that nerve function may alter over time.

The VibroSense Meter has shown promising results on the association between high VPTs and the risk of foot ulcers, as well as with difficulties in balance and gait and weakness of the foot, in adult T1D subjects.[22] However, it has not been tested for these parameters in paediatric subjects. It would be very hard to carry out similar studies in paediatric subjects since the above-mentioned symptoms and complications to DPN are very rare in paediatric T1D subjects.[10] Furthermore, potential differences in central and peripheral plasticity and neurogenesis between paediatric, particularly among the younger ones, and adult subjects must be taken into account.[23] A study by Selvarajah et al. suggests that DPN is not only associated with damage to the peripheral nerves, but also with morphological changes to the primary sensory cortex.[24] Moreover, previous studies have shown that children under 12 years of age undergoing peripheral nerve repair have better functional recovery 30 years post-injury and repair than adolescents (12–20 years of age) due to cerebral plasticity.[25] With this in mind, it is likely to believe that, depending on at what age the peripheral nervous system is damaged, there might be different recovery responses.

A limitation to our study is the sample size and the relatively large number of non-attenders compared to baseline. The characteristics of the non-attenders at baseline, i.e., higher HbA1c compared to attenders, could possibly reflect worse glucose control, than among attenders, and consequently impaired vibrotactile sense. To be able to make clear conclusions of how DPN develops over time in paediatric T1D subjects, a larger cohort would be necessary, as well as repeated vibrotactile measurements over a longer period. Another limitation is that the VibroSense Meter is not validated in children in comparison with nerve conduction studies. However, a strength of the study is the large normal material previously collected,[14] to which z-scores could be calculated and related to. The lack of a comprehensive clinical neurological examination of the subjects must also be stated as a limitation. However, it is unlikely to find signs of clinical DPN in this cohort since DPN in paediatric T1D subjects is most commonly subclinical, and the subjects' medical records have been thoroughly screened for clinical signs of DPN.[3]

The advantage of z-transformation is that the VPTs are not only compared between subjects with T1D, but also to healthy controls. However, a limitation is that some individuals have, due to increased age, changed comparison groups between baseline and follow-up. We have chosen to use the correct comparison-group based on their actual age, and not the group that was used at baseline. We believe that, due to a strong normal material, this will more truly reflect the vibrotactile sense at the age of follow-up, but since it is open for debate, we would like to claim this as a limitation.

Although there is no current treatment for DPN today, it is important to raise awareness of DPN and other complications early in the course of the subject's disease. An early detection of impaired sense makes it possible to work proactively in order to minimize the risk of developing diabetic foot ulcers later on.

In future studies with the VibroSense Meter, it would be of great interest to include subjects in a prospective study, where they undergo repeated examinations, for example, with the first examination shortly after T1D onset in order to see how the vibrotactile sense develop over time.

In conclusion, the current study indicates that the vibrotactile sense might change over time in paediatric T1D subjects, but larger studies, and studies with a normal group of healthy individuals followed-up during the same time, are needed to establish these findings.

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CONFLICT OF INTEREST

Nothing to declare.

CONSENTS

Written informed consents to participate, and that data would be published, were obtained from all subjects as well as their legal guardians.

DATA AVAILABILITY STATEMENT

Public access to data is restricted by the Swedish Authorities (Public Access to Information and Secrecy Act; https://www.government.se/information-material/2009/09/public-access-to-information-and-secrecy-act/), but data can be made available for researchers after a special review that includes approval of the research project by both an Ethics Committee and the authorities' data safety committees.

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SUPPORTING INFORMATION

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DIABETIC Medicine

RESEARCH: COMPLICATIONS

Quantitative proteomic analysis of human peripheral nerves from subjects with type 2 diabetes

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Abstract

Aims: Diabetic peripheral neuropathy (DPN) is a common and severe complication to type 2 diabetes. The pathogenesis of DPN is not fully known, but several pathways and gene polymorphisms contributing to DPN are described. DPN can be studied using nerve biopsies, but studies on the proteome of the nerve itself, and its surrounding tissue as a whole, are lacking. Studies on the posterior interosseous nerve (PIN) have proposed PIN a useful indicator of DPN.

Methods: A quantitative mass spectrometry-based proteomics analysis was made of peripheral nerves from age- and gender-matched living human male tissue donors; nine type 2 diabetes subjects, with decreased sural nerve action potentials indicating DPN, and six controls without type 2 diabetes, with normal electrophysiology results.

Results: A total of 2617 proteins were identified. Linear regression was used to discover which proteins were differentially expressed between type 2 diabetes and controls. Only soft signals were found. Therefore, clustering of the 500 most variable proteins was made to find clusters of similar proteins in type 2 diabetes subjects and healthy controls.

Conclusions: This feasibility study shows, for the first time, that the use of quantitative mass spectrometry enables quantification of proteins from nerve biopsies from subjects with and without type 2 diabetes, which may aid in finding biomarkers of importance to DPN development.

KEYWORDS
diabetes mellitus, diabetic neuropathies, peripheral neuropathies, proteome, type 2

1 | INTRODUCTION

The prevalence of type 2 diabetes is increasing worldwide.[1] Microvascular complications of long-term diabetes involve both the autonomic and peripheral nervous systems and present clinical manifestations, such as cardiovascular autonomic neuropathy and diabetic peripheral neuropathy (DPN).[1] Although DPN is a well-known and devastating complication, much of its pathogenesis remains unexplained, although genetic risk profile genes, and several signal pathways and cascades, have been highlighted for type 2 diabetes in DPN.[1] While promising data have been presented on possible treatments of DPN in animal models, like aldose reductase inhibitors, results have failed to be reproduced in human subjects.[1]

To the best of our knowledge, no studies have been done using mass spectrometry on human peripheral nerve tissue from type 2 diabetes subjects to establish the protein profiles of the tissue. Proteomics have been used to study peripheral nerve regeneration and repair, mainly focusing on Schwann cells, in studies using tissue obtained in reconstructive surgery, amputations and organ donations, but not specifically in subjects with type 2 diabetes.[2]

Using human nerve biopsies to study the development of DPN would be beneficial since animal models have been shown to have difficulties in mimicking the neuropathic environment in humans.[3] Furthermore, mass spectrometry enables screening of a large number of proteins in the nerve biopsies in contrast to immunocytochemical staining of present specific proteins, which, however, allow topographical tissue localization of the proteins.

The human posterior interosseous nerve (PIN) is a peripheral nerve from the deep branch of the radial nerve located in the dorsal aspect of the forearm.[3] The most distal part of the PIN is solely sensory, like the sural nerve of the lower extremity. PIN has been proposed as an indicator of DPN, being able to determine differences in myelinated nerve fibre density (MNFD) in subjects with type 2 diabetes, compared to post-mortem controls, as well as showing differences in MNFD between subjects with and without diabetes with carpal tunnel syndrome.[3,4]

The current feasibility study primarily aims to present quantitative mass spectrometry data from human peripheral nerves obtained from elderly male subjects with and without type 2 diabetes. We discuss our findings in light of described pathogenesis pathways[1] in DPN and furthermore elaborate our thoughts about identified proteins of interest to nerve damage and repair.

Novelty statement

- This study presents, for the first time, the full proteome of full nerve tissues of the posterior interosseus nerve, a peripheral nerve in the forearm, obtained from healthy subjects and subjects with type 2 diabetes and signs of diabetic peripheral neuropathy (DPN).
- Proteins, either known to or expected to contribute to DPN development, are discussed in a descriptive manner.
- The proteins are not presented as potential biomarkers of DPN, but rather chosen to enlighten the possibilities of a proteomic approach to study DPN in the future.

2 | PARTICIPANTS AND METHODS

2.1 | Subjects

PIN biopsies were performed in a total of 15 subjects (type 2 diabetes: $n = 9$; healthy controls: $n = 6$). type 2 diabetes subjects were recruited from a cohort, originally participating in a prospective health screening study in Malmö, Sweden.[5] Half of the controls ($n = 3$) were recruited from the same study as type 2 diabetes subjects, and the other half ($n = 3$) were recruited from a study on, otherwise healthy, subjects with carpal tunnel syndrome (CTS) undergoing carpal tunnel release with concurrent PIN biopsy.[4] Thus, type 2 diabetes subjects were compared with age- and gender-matched healthy controls.

2.2 | Electrophysiology

Sural nerve sensory conduction velocity (sSCV) and sural nerve amplitude (sSAMP), as well as peroneal motor conduction velocity (pMCV), were obtained from the lower extremity. Z-scores of sSCV, sSAMP and pMCV were calculated using normative data previously collected and published with negative z-scores indicating DPN (see supplement).

Sensory and motor electrophysiology data, including z-scores based on normal material from the investigating laboratory, of the ulnar nerve obtained from the same arm as PIN biopsy are presented. The sensory parameters are ulnar sensory amplitude (uSAMP) and ulnar sensory conduction velocity (uSCV) at wrist level. Ulnar motor parameters are ulnar motor conduction velocity (uMCV), ulnar motor amplitude at wrist level (uAMP wrist) and ulnar distal motor latency (uDML).

2.3 | Posterior interosseous nerve

The PIN is a non-compressed nerve at the distal, dorsal side of the forearm. PINs were harvested under local or regional anaesthesia, as described by Thomsen et al.[3] The harvested specimen, around 3–4 cm of length, was cut into four equally long pieces for different analyses; the present study and two other studies presented in the supplement to this article. The weight of the nerve specimen is 1.16 mg per mm, and each specimen used in this study was 5 mm long, that is, 5.8 mg each.

2.4 | MS sample preparation

Fresh frozen tissue pieces were suspended in an extraction buffer with 100 mM ammonium bicarbonate with 8 M urea

and homogenized using a Bioruptor® Plus (Diagenode SA, Seraing, Belgium) at 4°C for 40 cycles 15 s ON/OFF. Samples were reduced with 5 mM TCEP (tris-2-carboxyethyl phosphine) 30 min at 37°C at 850 rpm, alkylated with 10 mM IAA (iodoacetamide) for 45 min at room temperature in the dark. The extracted proteins were digested overnight using 1 μg of trypsin per sample at 37°C. Digested samples were centrifuged for 10 min at 14 000 g prior to desalting using SOLAμ™-SPE plates (Thermo Scientific) according to the manufacturer's instructions. Desalted samples were dried and resuspended in 2% acetonitrile and 0.1% formic acid. The peptide concentration of the samples was measured after digestion using Pierce colorimetric peptide assay #23275 (Thermo Fisher Scientific).

2.5 | LC-MS/MS analysis

Peptides were separated on an EASY-nLC 1000 HPLC system (Thermo Fisher Scientific, Bremen, Germany) connected to a 25 cm EASY-Spray column PepMap®RSLC C18 (Thermo Fisher Scientific). For data-dependent acquisition (DDA), an LC-gradient of 60 min was used, whereas for data-independent acquisitions (DIA) the LC-gradient was extended to 120 min. For both acquisition types, a gradient of 5%–30% solvent B (0.1% formic acid and 100% acetonitrile) over 60 or 120 min was followed by 5 min increase to 95% solvent B, at a flow rate of 300 nl/min. All data acquisitions were performed on a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific). For DDA analyses each survey scan (resolution 70,000 at 200 m/z) at mass range 400–1600 was followed by MS/MS scans of the top 15 most intense ions. For DIA, MS survey scans at mass range 400–1200 m/z were followed by 32 MS/MS full fragmentation scans with an isolation window of 26 m/z as described in Malmström et al. and Teleman et al.[6,7]

2.6 | Mass spectrometry data analysis

MS raw data were converted to gzipped and Numpressed mzML[8] using the tool MSconvert from the ProteoWizard, v3.0.5930 suite.[9] All data analyses were stored and managed using openBIS.[10] The DDA data acquired spectra were analysed using the search engine X! Tandem (2013.06.15.1-LabKey, Insilicos, ISB),[11] OMSSA (version 2.1.8)[12] and COMET (version 2014.02 rev.2) against an in-house compiled database containing the *Homo sapiens* reference proteome (UniProt proteome IDs UP000005640) supplemented with common contaminants. Fully tryptic digestion was used allowing two missed cleavages. Carbamidomethylation (C) was set to static and oxidation (M) to variable modifications, respectively. Mass tolerance for precursor ions was set to 0.2 Da, and for fragment ions to 0.02 Da. Identified peptides were processed and analysed through the Trans-Proteomic Pipeline (TPP v4.7 POLAR VORTEX rev 0, Build 201403121010) using PeptideProphet.[13] The false discovery rate (FDR) was estimated with Mayu (version 1.07) and peptide spectrum matches (PSMs) were filtered with protein FDR set to 1% resulting in a peptide FDR <1%.

The DIA data were processed using the OpenSWATH pipeline.[14] For DIA data analysis, spectral libraries from the above DDA dataset were created in openBIS[10] using SpectraST (version 5.0, TPP v4.8.0 PHILAE, build 201506301157-exported (Ubuntu-×86_64)) in TPP.[15] For DIA data analysis, raw data files were converted to mzXML using msconvert and analysed using OpenSWATH (version 2.0.1 revision: c23217e). The RT extraction window was ±300 s, and m/z extraction was set at 0.05 Da tolerance. RT was then calibrated using iRT peptides. Peptide precursors were identified by OpenSWATH (2.0.1) and PyProphet (2.0.1) was used to control the false discovery rate of 1% at peptide precursor level and at 1% at protein level. Then TRIC[16] was used to align the runs in the retention time dimension and reduce the identification error by decreasing the number of missing values in the quantification matrix. Further missing values were re-quantified by TRIC.[16] Resulting DIA datasets were analysed using Jupyter Notebooks (version 3.1.1). Relative protein abundancy was estimated by summing the intensities of the most intense three peptides for each protein relative to the total peptide intensities (without iRT) for that protein. The resulting output data were normalized using the total-ion count (TIC) to correct for slight variations in sample amounts.

2.7 | Statistical analyses

Subject characteristics are presented as medians [quartiles]. Differences between type 2 diabetes subjects and controls are calculated using Mann–Whitney U-test, with p-values <0.05 considered significant. Statistical calculations of characteristics are made using IBM SPSS Statistics version 26 for Mac (SPSS Inc.).

In order to control for confounders, a linear regression model was used on electrophysiology data that significantly differed between type 2 diabetes and controls. Z-score was used as dependent variable and type 2 diabetes, age and BMI as independent variables.

The individual protein intensities obtained from biopsies of the 15 subjects were log2-transformed and normalized. Linear regression was used to discover which proteins were differentially expressed between type 2 diabetes and controls. Since no significant differences were found between the groups, the 500 proteins with the highest variance

across all samples were selected for additional analysis and no further comparisons between the groups were made regarding protein intensities.[17] Unsupervised hierarchical clustering with complete linkage was performed on the 500 proteins with the highest variance across all samples in order to find clusters of similar proteins. All statistical protein analyses were performed in R version 3.5.1 (R core team, R Foundation for Statistical Computing).

2.8 | Protein interactions

The STRING database version 11 was used to graphically present potential linkages and interactions between identified proteins.[18] STRING database consists of known, as well as expected, protein–protein interactions. The known and potential protein–protein interactions are presented graphically, and the level of evidence connecting the proteins is represented by connecting lines of different colour (see results).[18]

2.9 | Gene ontology

Gene ontology (GO) was used to visualize clustered proteins in terms of enriched GO terms, based on biological process, molecular function and cellular component, as described by the Gene Ontology Consortium.[19–21] Corrections for multiple testing were done using false discovery rate and p-values <0.05 were considered statistically significant.

2.10 | Ethics

Ethical permit has been granted by the local ethics committee at Lund University, Lund, Sweden (LU508-03 and LU504-03). The research is conducted in accordance with the declaration of Helsinki and written inform consent was obtained from all subjects.

3 | RESULTS

3.1 | Subjects

Characteristics of the subjects ($n = 15$) are presented in Table 1, previously published by Thomsen et al. and Ekman et al.[3,4,22] Median age [quartiles] was 74 [74–77] and 72 [68–75] for subjects with ($n = 9$) and without ($n = 6$) diabetes, respectively ($p = 0.04$; Table 1). Median

TABLE 1 Characteristics of subjects

	Type 2 diabetes ($n = 9$)	Controls ($n = 6$)	All ($n = 15$)	Type 2 diabetes vs Control
	Median [quartiles]	Median [quartiles]	Median [quartiles]	p-value
Characteristics				
Age (years)	74 [74–77]	72 [68–75]	74 [72–77]	**0.040**
Height (cm)	176 [171–178]	177 [172–180]	176 [173–178]	0.478
Weight (kg)	82 [68–87]	88 [80–102]	82 [79–93]	0.289
BMI	26.4 [24.5–27.6]	28.3 [25.9–34.3]	26.5 [26.2–29.2]	0.140
HbA_{1c} (mmol/mol)	40 [29–62]	29 [26–32]	32 [28–54]	**0.028**
(HbA_{1c} (%))	(5.8 [4.8–7.8])	(4.75 [4.5–5.1])	(5.1 [4.7–7.1])	
P-cholesterol (mmol/L)	3.9 [3.3–5.0]	5.0 [4.0–5.0][a]	4.4 [3.8–5.0][b]	0.224
P-HDL (mmol/L)	1.1 [1.0–1.7]	1.3 [1.1–1.3][a]	1.2 [1.0–1.8][b]	0.308
P-LDL (mmol/L)	2.4 [1.9–2.9]	2.9 [1.8–2.9][a]	2.5 [1.9–3.0][b]	0.643
LDL/HDL	2.0 [1.4–2.4]	1.6 [1.5–1.6][a]	1.9 [1.4–2.3][b]	0.926
P-TG (mmol/L)	1.0 [0.9–1.8]	1.5 [0.9–1.5][a]	1.1 [0.9–1.8][b]	0.513

Characteristics are presented as median [quartiles] for type 2 diabetes and controls, respectively, and for the whole group, and comparisons between type 2 diabetes and controls are made using Mann–Whitney U-tests. Data on lipid status were missing in three controls. Data previously published by Thomsen et al. and Ekman et al.[3,4,22]

[a] $n = 3$.

[b] $n = 12$.

HbA_{1c} was significantly higher among type 2 diabetes subjects compared to control, 40 [29–62] mmol/mol (5.8 [4.8–7.8] %) vs. 29 [26–32] mmol/mol (4.8 [4.5–5.1] %) ($p = 0.028$; Table 1).

All but one type 2 diabetes subjects had disease duration of at least 15 years. The type 2 diabetes subject with disease duration less than 15 years had impaired glucose tolerance (IGT) 12 years prior to type 2 diabetes diagnosis, indicating pre-diabetes. At least two type 2 diabetes subjects were treated with insulin at time of biopsy, and two subjects were treated with oral antidiabetic agents; incomplete data in $n = 5$ who at least were treated with oral antidiabetic agents.

3.2 | Electrophysiology and PIN morphometry

Electrophysiology, from upper and lower extremities, and PIN morphometry data, previously published by Thomsen et al., Osman et al. and Ekman et al.,[3,4,22,23] are presented in Table 2. sSCV, sSAMP and pMCV were overall 41.5 (39.8–44.0), 4.0 (2.0–6.0) and 40.0 [34.0–46.0], respectively, and not significantly different regarding sSCV ($p = 0.896$) or pMCV ($p = 0.173$). However, sSAMP was significantly lower ($p = 0.032$) among type 2 diabetes subjects compared to controls.[3,4,22,23] Z-scores of sSCV and pMCV were not significantly different between type 2 diabetes subjects

TABLE 2 Nerve function and morphometrics

	Type 2 diabetes ($n = 9$)	Controls ($n = 6$)	All ($n = 15$)	Type 2 diabetes vs Control
	Median [quartiles]	Median [quartiles]	Median [quartiles]	*p*-value
Electrophysiology				
sSCV (m/s)	42.0 [40.0–43.8][a]	41.0 [39.0–45.5]	41.5 [39.8–44.0][b]	0.896
Z-score sSCV	−1.3 [−1.8–−0.9][a]	−1.6 [−2.0–−0.5]	−1.5 [−1.9–−0.9][b]	0.896
sSAMP (μV)	2.0 [1.5–4.5][a]	4.5 [4.0–6.8]	4.0 [2.0–6.0][b]	**0.032**
Z-score sSAMP	−1.5 [−2.0–−0.5][a]	−0.6 [−0.8–0.4]	−0.8 [−1.9–−0.3][b]	**0.039**
pMCV (m/s)	37.0 [33.5–44.0]	44.0 [38.3–47.0]	40.0 [34.0–46.0]	0.173
Z-score pMCV	−1.7 [−3.2–0.6]	0.3 [−1.4–1.5]	−0.7 [−2.9–1.3]	0.262
uSCV (m/s)	48.0 [42.8–48.8][c]	54.0 [46.5–55.0][d]	48.0 [45.5–52.0][e]	0.140
Z-score uSCV	0.2 [−0.5–0.4][c]	0.9 [−0.1–1.2][d]	0.2 [−0.2–0.7][e]	0.143
uSAMP (μV)	3.0 [2.0–4.0][c]	4.0 [3.0–4.5][d]	3.0 [2.5–4.0][e]	0.255
Z-score uSAMP	0.0 [−0.7–0.6][c]	0.1 [−0.3–0.7][d]	0.0 [−0.6–0.6][e]	0.607
uMCV (m/s)	56.0 [50.5–57.8][c]	52.0 [48.5–57.0][d]	54.0 [50.5–57.5][e]	0.509
Z-score uMCV	0.6 [−0.3–0.8][c]	−0.3 [−0.8–0.7][d]	0.2 [−0.4–0.8][e]	0.380
uAMP wrist (μV)	5.7 [5.0–6.8][c]	6.9 [5.5–7.4][d]	5.7 [5.3–7.1][e]	0.372
Z-score uAMP wrist	−0.4 [−0.8–0.3][c]	0.2 [−0.5–0.5][d]	−0.4 [−0.6–0.4][e]	0.509
uDML (ms)	3.3 [3.1–3.5][c]	3.5 [3.1–3.7][d]	3.3 [3.2–3.5][e]	0.372
Z-score uDML	−0.2 [−0.8–0.4][c]	0.6 [−0.7–1.0][d]	−0.2 [−0.7–0.5][e]	0.242
Morphometrics				
PIN MNFD (numbers/mm^2)	5432 [4762–7116]	4961 [4187–5172]	5116 [4733–6133]	0.099
Proteomics				
No of proteins	2555 [2538–2567]	2521 [2507–2553]	2543 [2520–2565]	0.099

Data are presented as median (quartiles) for type 2 diabetes and controls, respectively, and for the whole group and comparisons are made between type 2 diabetes subjects and controls using Mann–Whitney U-tests. Data previously published (except proteomics data) by Thomsen et al., Osman et al. and Ekman et al.[3,4,22,23]

sSCV sural sensory conduction velocity, sSAMP sural sensory amplitude, pMCV peroneal motor conduction velocity, uSCV ulnar sensory conduction velocity, uSAMP ulnar sensory amplitude, uMCV ulnar motor conduction velocity, uAMP wrist ulnar motor amplitude at wrist level, uDML ulnar distal motor latency.

[a] $n = 8$.

[b] $n = 14$.

[c] $n = 8$.

[d] $n = 5$.

[e] $n = 13$.

and controls ($p = 0.896$ and $p = 0.262$, respectively), but Z-score of sSAMP was significantly lower among type 2 diabetes subjects ($p = 0.039$), indicating early signs of impaired nerve function. Using a linear regression model showed that z-scores of sSAMP were lower among type 2 diabetes subjects compared to controls (B: −1.203 [−2.218 - −0.189]; $p = 0.024$) and that sSAMP did not change when age (B: 0.074 [−0.118–0.265]; $p = 0.415$) and BMI (B: −0.023 [−0.250–0.205]; $p = 0.829$ were added to the regression model.

Electrophysiology from the ulnar nerve including z-scores, presented in Table 2, did not differ at any parameter between type 2 diabetes subjects compared to controls.

MNFD was not significantly different between type 2 diabetes and control subjects, 5432 [4762–7116] vs 4961 [4187–5172] ($p = 0.099$); data previously published in Thomsen et al and Osman et al.[3,4,23]

3.3 | Proteomics

The mass spectrometry analysis enabled quantification of median 2543 proteins (Table 2) in PIN nerves at a false discovery rate of 1%, with a total of 2617 different proteins identified on group level (Table S2). As mentioned, since no significant differences were found between groups when using linear regression to discover differentially expressed proteins between type 2 diabetes and controls, the 500 most variable proteins were clustered, as described in the Methods section, and presented graphically in Figure 1 and as a zoomable version in Figure S1. No definitive clustering was found, but rather soft signals. A heat map showing the variability among the clustered proteins, grouped into three clusters, is presented in Figure 1. The first cluster consists of 72 proteins and is also presented in Table 3 as well as graphically with potential interactions and linkages, according to STRING database version 11, in Figure 2. Out of 72 proteins in cluster 1, 58 were recorded in STRING Database version 11. Clusters 2 and 3 consist of 63 and 365 proteins, respectively, and are presented in Table S1. All 2617 identified proteins are presented in full in Table S2.

In Figure 2, three distinct groups of proteins appear, where the histone group is the largest. Furthermore, collagens are grouped together with metalloproteinase inhibitor-3 (TIMP3) and haemoglobin subunits constitutes one group. In addition to these groups, protein–protein interactions are seen between myelin basic protein (MBP), myelin protein P0 (MPZ) and glial fibrillary acidic protein (GFAP), as well as between apolipoprotein C-III (APOC3) and apolipoprotein(a) (Apo(a)), the main constituent of Lipoprotein(a) (Lp(a)).

The 20 most enriched GO terms in each of the following categories, biological process (blue), molecular function (orange) and cellular component (green) are presented in Figure 3.

The protein abundancy for some of the proteins, selected because of known and expected relevance to DPN, is shown grouped using String Database, for the 72 most variable proteins. The protein intensities are presented as boxplots, for type 2 diabetes subjects and controls separately, in Figures 4 and 5.

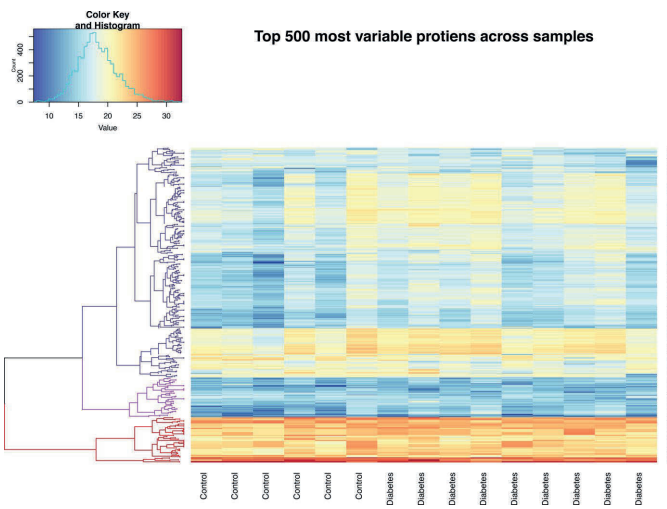

FIGURE 1 Heat map of the 500 most variable proteins clustered. Heat map showing the variability of proteins among subjects and controls. In the left margin, the clusters from Figure 1 are shown; red is cluster 1, purple is cluster 2 and blue is cluster 3. The number in the right margin corresponds to the identification number in Table S1. Please see zoomable version of the clustering in Figure S1 attached

TABLE 3 All 72 identified proteins from cluster 1 are presented with full name, gene and UniProt code

Protein	Gene	UniProt	Protein	Gene	UniProt
CO1A1_HUMAN|Collagen alpha−1(I) chain	**COL1A1**	**P02452**	Immunoglobulin heavy chain variable region	Unknown	Unknown
CO1A2_HUMAN|Collagen alpha−2(I) chain	**COL1A2**	**P08123**	HV353_HUMAN|Immunoglobulin heavy variable 3–53	IGHV3-53	P01767
HBA_HUMAN|Haemoglobin subunit alpha	HBA1	P69905	CBPA3_HUMAN|Mast cell carboxypeptidase A	CPA3	P15088
HBB_HUMAN|Haemoglobin subunit beta	HBB	P68871	F171B_HUMAN|Protein FAM171B	FAM171B	Q6P995
MBP_HUMAN|Myelin basic protein	**MBP**	**P02686**	H2A1D_HUMAN|Histone H2A type 1-D	HIST1H2AD	P20671
HBAZ_HUMAN|Haemoglobin subunit zeta	HBZ	P02008	H2A1_HUMAN|Histone H2A type 1	HIST1H2AG	P0C0S8
MYP0_HUMAN|Myelin protein P0	**MPZ**	**P25189**	H2A1H_HUMAN|Histone H2A type 1-H	HIST1H2AH	Q96KK5
HBD_HUMAN|Haemoglobin subunit delta	HBD	P02042	H2A1J_HUMAN|Histone H2A type 1-J	HIST1H2AJ	Q99878
CO3A1_HUMAN|Collagen alpha−1(III) chain	**COL3A1**	**P02461**	H2AJ_HUMAN|Histone H2A.J	H2AFJ	Q9BTM1
CAH1_HUMAN|Carbonic anhydrase 1	CA1	P00915	H2B2C_HUMAN|Putative histone H2B type 2-C	HIST2H2BC	Q6DN03
CLIC1_HUMAN|Chloride intracellular channel protein 1	CLIC1	O00299	H2B2D_HUMAN|Putative histone H2B type 2-D	HIST2H2BD	Q6DRA6
GFAP_HUMAN|Glial fibrillary acidic protein	**GFAP**	**P14136**	RAB2A_HUMAN|Ras-related protein Rab−2A	RAB2A	P61019
TBB1_HUMAN|Tubulin beta−1 chain	**TUBB1**	**Q9H4B7**	CARD6_HUMAN|Caspase recruitment domain-containing protein 6	CARD6	Q9BX69
TARSH_HUMAN|Target of Nesh-SH3	ABI3BP	Q7Z7G0	**PEPL_HUMAN|Periplakin**	**PPL**	**O60437**
NUP93_HUMAN|Nuclear pore complex protein Nup93	NUP93	Q8N1F7	A2ML1_HUMAN|Alpha−2-macroglobulin-like protein 1	A2ML1	A8K2U0
MYG_HUMAN|Myoglobin	MB	P02144	H2A3_HUMAN|Histone H2A type 3	HIST3H2A	Q7L7L0
BASP1_HUMAN|Brain acid soluble protein 1	BASP1	P80723	H2A1B_HUMAN|Histone H2A type 1-B/E	HIST1H2AB	P04908
B5MDG6_HUMAN|Protein POM121L7	POM121L7	B5MDG6	$H2A_{1C}$_HUMAN|Histone H2A type 1-C	HIST1H2AC	Q93077
P12L1_HUMAN|Putative POM121-like protein 1	POM121L1P	Q3SYA9	**ANXA3_HUMAN|Annexin A3**	**ANXA3**	**P12429**
TBA4B_HUMAN|Putative tubulin-like protein alpha−4B	TUBA4B	Q9H853	H2AX_HUMAN|Histone H2AX	H2AFX	P16104
KCRM_HUMAN|Creatine kinase M-type	CKM	P06732	H2A2B_HUMAN|Histone H2A type 2-B	HIST2H2AB	Q8IUE6
H2A2A_HUMAN|Histone H2A type 2-A	HIST2H2AA3	Q6FI13	H15_HUMAN|Histone H1.5	HIST1H1B	P16401
H2A2C_HUMAN|Histone H2A type 2-C	HIST2H2AC	Q16777	**S27A5_HUMAN|Bile acyl-CoA synthetase**	**SLC27A5**	**Q9Y2P5**

(Continues)

TABLE 3 (Continued)

Protein	Gene	UniProt	Protein	Gene	UniProt
HBG2_HUMAN|Haemoglobin subunit gamma−2	HBG2	P69892	**TIMP3_HUMAN|Metalloproteinase inhibitor 3**	**TIMP3**	**P35625**
Ig kappa chain V region L25	Unknown	Unknown	LDH6B_HUMAN|L-lactate dehydrogenase A-like 6B	LDHAL6B	Q9BYZ2
KV315_HUMAN|Immunoglobulin kappa variable 3–15	IGKV3-15	P01624	**APOC3_HUMAN|Apolipoprotein C-III**	**APOC3**	**P02656**
HBG1_HUMAN|Haemoglobin subunit gamma−1	HBG1	P69891	BBS7_HUMAN|Bardet-Biedl syndrome 7 protein	BBS7	Q8IWZ6
HV169_HUMAN|Immunoglobulin heavy variable 1–69	IGHV1-69	P01742	41_HUMAN|Protein 4.1	EPB41	P11171
HV146_HUMAN|Immunoglobulin heavy variable 1–46	IGHV1-46	P01743	MAP2_HUMAN|Methionine aminopeptidase 2	METAP2	P50579
HV103_HUMAN|Immunoglobulin heavy variable 1–3	IGHV1-3	A0A0C4DH29	BAF_HUMAN|Barrier-to-autointegration factor	BANF1	O75531
A0A0B4J2H0_HUMAN Protein IGHV1-69–2 (Fragment)	IGHV1-69–2	A0A0B4J2H0	H2AV_HUMAN|Histone H2A.V	H2AFV	Q71UI9
COIA1_HUMAN|Collagen alpha−1(XVIII) chain	**COL18A1**	**P39060**	H2AZ_HUMAN|Histone H2A.Z	H2AFZ	P0C0S5
CTTB2_HUMAN|Cortactin-binding protein 2	CTTNBP2	Q8WZ74	HINT1_HUMAN|Histidine triad nucleotide-binding protein 1	HINT1	P49773
HV321_HUMAN|Immunoglobulin heavy variable 3–21	IGHV3-21	A0A0B4J1V1	GTR1_HUMAN|Solute carrier family 2, facilitated glucose transporter member	SLC2A1	P11166
HV311_HUMAN|Immunoglobulin heavy variable 3–11	IGHV3-11	P01762	**APOA_HUMAN|Apolipoprotein(a)**	**LPA**	**P08519**
HV333_HUMAN|Immunoglobulin heavy variable 3–33	IGHV3-33	P01772	KVD20_HUMAN|Immunoglobulin kappa variable 3–20	IGKV3D−20	A0A0C4DH25

Proteins in **bold** are presented separately in Figures 4 and 5 with boxplots showing the difference of the quantified proteins between type 2 diabetes subjects and controls.

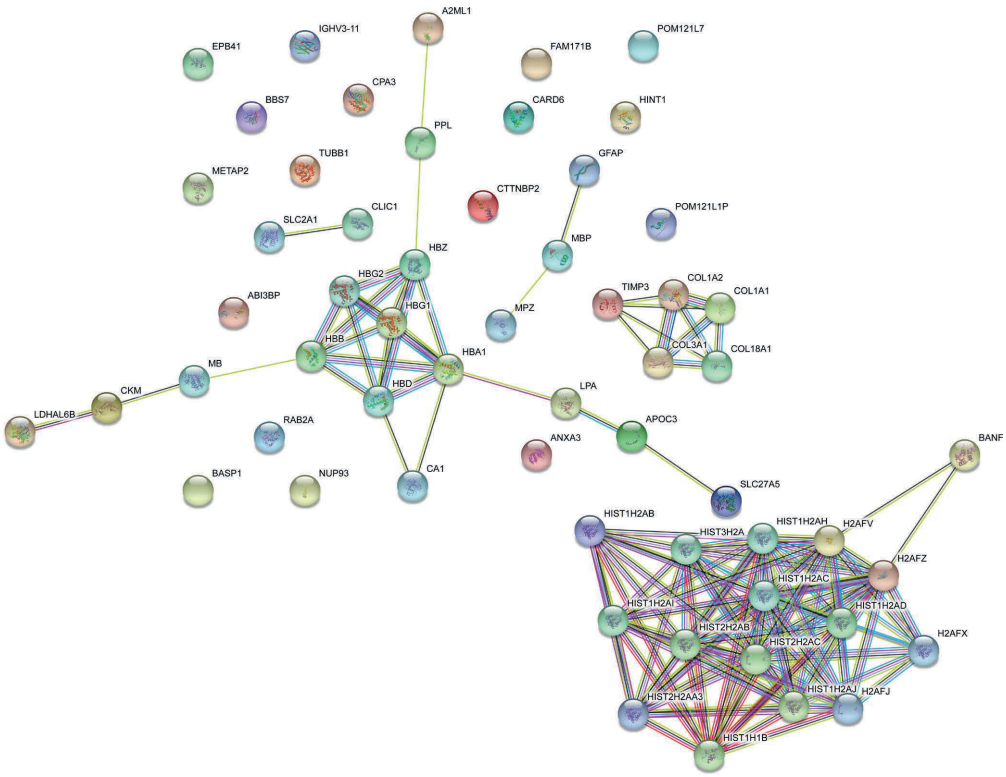

FIGURE 2 Interactions map. The protein interactions are presented graphically. Nodes are connected with lines of different colours depending on the level of scientific evidence connecting them. Known interactions are indicated with either turquoise (from curated databases) or pink (experimentally determined) edges, whereas predicted interactions are shown with green (gene neighbourhood), red (gene fusions) or blue (gene co-occurrence) edges. Other potential interactions are shown in light green (text mining), black (co-expression) and blue/white (protein homology).[18]

4 | DISCUSSION

In this study, we approach DPN, a well-known complication to type 2 diabetes, from a new perspective. We use quantitative mass spectrometry analysis on human peripheral nerve tissue from living type 2 diabetes subjects and, gender- and age-matched, controls in order to present the protein pattern of the entire nerve. Understanding a specific tissue's proteome could be essential when searching for disease biomarkers, as well as to understand the development of long-term complications. If proteins in the human nerves being of importance for the development of DPN can be identified, it might be possible in the future to screen for these proteins in blood samples instead of using nerve biopsies. However, in order to relate protein abundancy in the nerve to plasma levels of the same protein, and to clinical manifestations, larger study populations would be required.

We have chosen to present the 500 most variable proteins for the group, including both type 2 diabetes and control subjects. Since no significant differences were found between the groups using linear regression, no further comparisons were made statistically between the groups. We chose this approach in order to show the feasibility of identifying proteins that behave similar in type 2 diabetes and control subjects. Below we discuss a selection of proteins, either known to or expected to contribute to DPN development, in a descriptive manner. The proteins are not presented as potential biomarkers of DPN, but rather chosen to enlighten the possibilities of a proteomic approach to study DPN in the future.

FIGURE 3 GO enrichment terms. The 20 most enriched GO terms in each of the categories biological process (blue), molecular function (orange) and cellular component (green) are presented for the proteins of cluster 1 (see Table 3). From cluster 1, 69 out of 72 proteins were identifiable to GO

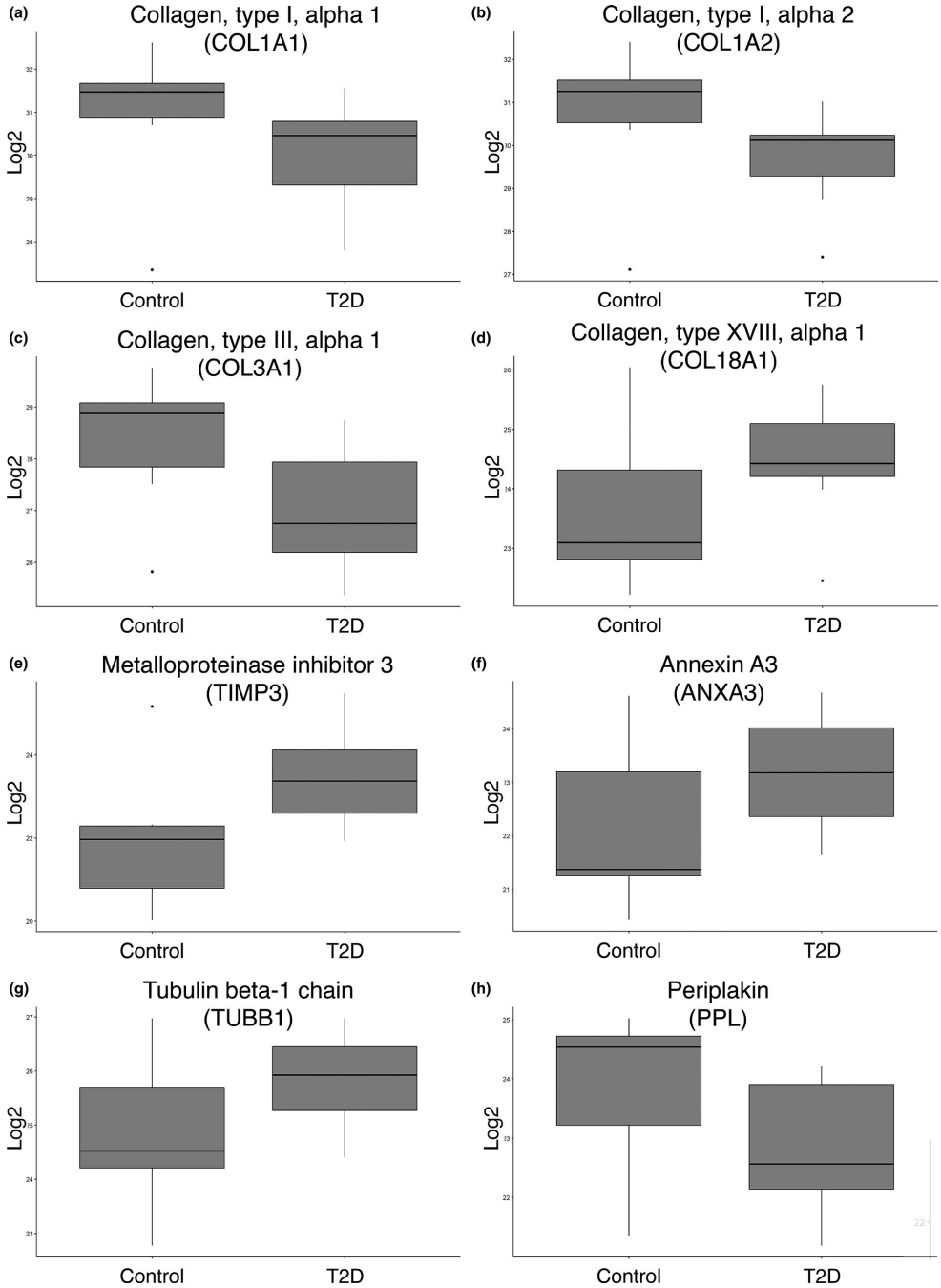
(a)
Collagen, type I, alpha 1
(COL1A1)
Log2
Control
T2D
(b)
Collagen, type I, alpha 2
(COL1A2)
Log2
Control
T2D
(c)
Collagen, type III, alpha 1
(COL3A1)
Log2
Control
T2D
(d)
Collagen, type XVIII, alpha 1
(COL18A1)
Log2
Control
T2D
(e)
Metalloproteinase inhibitor 3
(TIMP3)
Log2
Control
T2D
(f)
Annexin A3
(ANXA3)
Log2
Control
T2D
(g)
Tubulin beta-1 chain
(TUBB1)
Log2
Control
T2D
(h)
Periplakin
(PPL)
Log2
Control
T2D

FIGURE 4 Boxplots of protein intensities for proteins associated with extracellular matrix, cytoskeleton and neuropathic pain. (a) Collagen, type I, alpha 1 (COL1A1), (b) collagen, type I, alpha 2 (COL1A2), (c) collagen, type III, alpha 1 (COL3A1), (d) collagen, type XVIII, alpha 1 (COL18A1), (e) metalloproteinase inhibitor 3 (TIMP3), (f) annexin A3 (ANXA3), (g) tubulin beta-1 chain (TUBB1) and (h) periplakin (PPL). Boxplots show IQR of protein intensities with whiskers at 1.5× IQR and outliers ≤/≥ 1.5 × IQR

Among the most variable proteins in type 2 diabetes and control subjects were type I and type III collagens, known to constitute normal extracellular matrix comprising the peripheral nerve, as well as interacting with Schwann cells to regulate their function.[24] Our data suggest that both type I and type III collagens were more abundant in nerve biopsies from controls compared to type 2 diabetes subjects. This contrasts with findings by Bradley et al. of the extracellular matrix among type 1 diabetes and type 2 diabetes subjects, with confirmed sensory polyneuropathy and organ donor controls, where quantities of type I and type III collagens were increased in the endoneurium among subjects with diabetes compared to controls.[25] However, the results by Bradley et al. were based on both type 1 diabetes and type 2 diabetes subjects, with type 1 diabetes (mean age: 24.3 years, range: 23–26) subjects being markedly younger than type 2 diabetes subjects (mean age: 58.2 years [range: 31–75]).[25] Furthermore, we studied whole nerve biopsy samples while Bradley et al. specifically studied the endoneurium and were using qualitative morphological methods.[25]

In our study, type XVIII collagen was more abundant among type 2 diabetes subjects than controls. Type XVIII collagen can be cleaved into endostatin, a known angiogenesis inhibitor. It has also been described that higher levels of serum endostatin are strongly associated with kidney dysfunction and nephropathy in subjects with type 2 diabetes.[26] Potentially, higher abundancy of endostatin in type 2 diabetes subjects may play a role in development of microangiopathies, since endostatin acts as antiangiogenic.[26] Previous studies in cardiovascular research have suggested that higher circulating levels of endostatin might reflect vascular and myocardial damage.[26]

Among type 2 diabetes subjects, annexin A3 was more abundant compared to controls. Annexin A3 has been proposed to be of relevance in rats with induced neuropathic pain by chronic constriction injury (CCI) of a nerve.[27] Levels of annexin A3 were upregulated following the induction of neuropathic pain and pain was alleviated after administration of an agent downregulating annexin A3.[27] Although CCI, with its inherent less clinical relevance, does not provide the same neuropathic environment as DPN, other studies suggest an upregulated role of annexin A3 in microglia following axotomy, that is, a different kind of neural damage than CCI.[28] Being able to treat neuropathic pain in DPN would be of great importance since neuropathic pain is connected to great societal costs, as well as reduced quality of life.[1]

Several identified proteins, for example periplakin, were less abundant among type 2 diabetes subjects than controls. Periplakin is mostly described in diseases such as cancer and paraneoplastic pemphigus. Its actions are linked to the cytoskeletal organization, making it of interest to degeneration of nerve fibres in DPN.[29] A range of cytoskeletal proteins were identified in our samples, among them, in cluster 1, tubulin beta-1 chain; mostly associated with platelet dysfunction.[30] However, several other tubulins and other cytoskeletal proteins, for example tubulin beta-2 and −3 have also been identified in our material (Table S2). Microtubules, consisting of tubulins, have been shown to be of great importance in neurite outgrowth during nerve regeneration, and tubulin beta-2 and beta-3 have been shown to be of greater importance than tubulin beta-1 in neural regeneration following crush injury in rats.[1,31,32]

Furthermore, we identified several proteins connected to neuronal damage and myelination of peripheral nerves, for example MBP and MP0.[33] In addition, we noticed that GFAP was more abundant among type 2 diabetes subjects than controls. In several chronic neuropathies in humans, GFAP has been described as a marker for axonal damage, and elevated serum levels have been shown to correlate to decreased nerve action potentials.[34] Moreover, studies on mice suggest that defective GFAP might impair nerve regeneration in peripheral neuropathy.[35] In summary, the role of GFAP in the peripheral nerve is intriguing, since its abundancy appears to differ between type 2 diabetes subjects with significantly lower SAMPs compared to controls.

Identified proteins with great variability also include APOC3, closely linked to hypertriglyceridemia, and Apo(a), which together with a low-density lipoprotein-particle (LDL) compose Lp(a), known for its contribution to dyslipidaemia and atherosclerosis.[36] LDL oxidation mediates inflammation and reactive oxygen species (ROS) accumulation, leading to progressive nerve injury. We believe that our method of studying the neuronal proteome can be of use to more closely study the specific proteins engaged in these processes.[1]

Using mass spectrometry on PIN, not only nerve fibre tissue is analysed but also surrounding and supporting tissues, such as intraneural blood vessels, connective tissue components of the nerve and possibly adipose tissue remaining on the surface of the nerve even though the nerve was surgically 'cleaned' at harvest. Depending on how well the nerves are vascularized, a variable degree of plasma proteins will

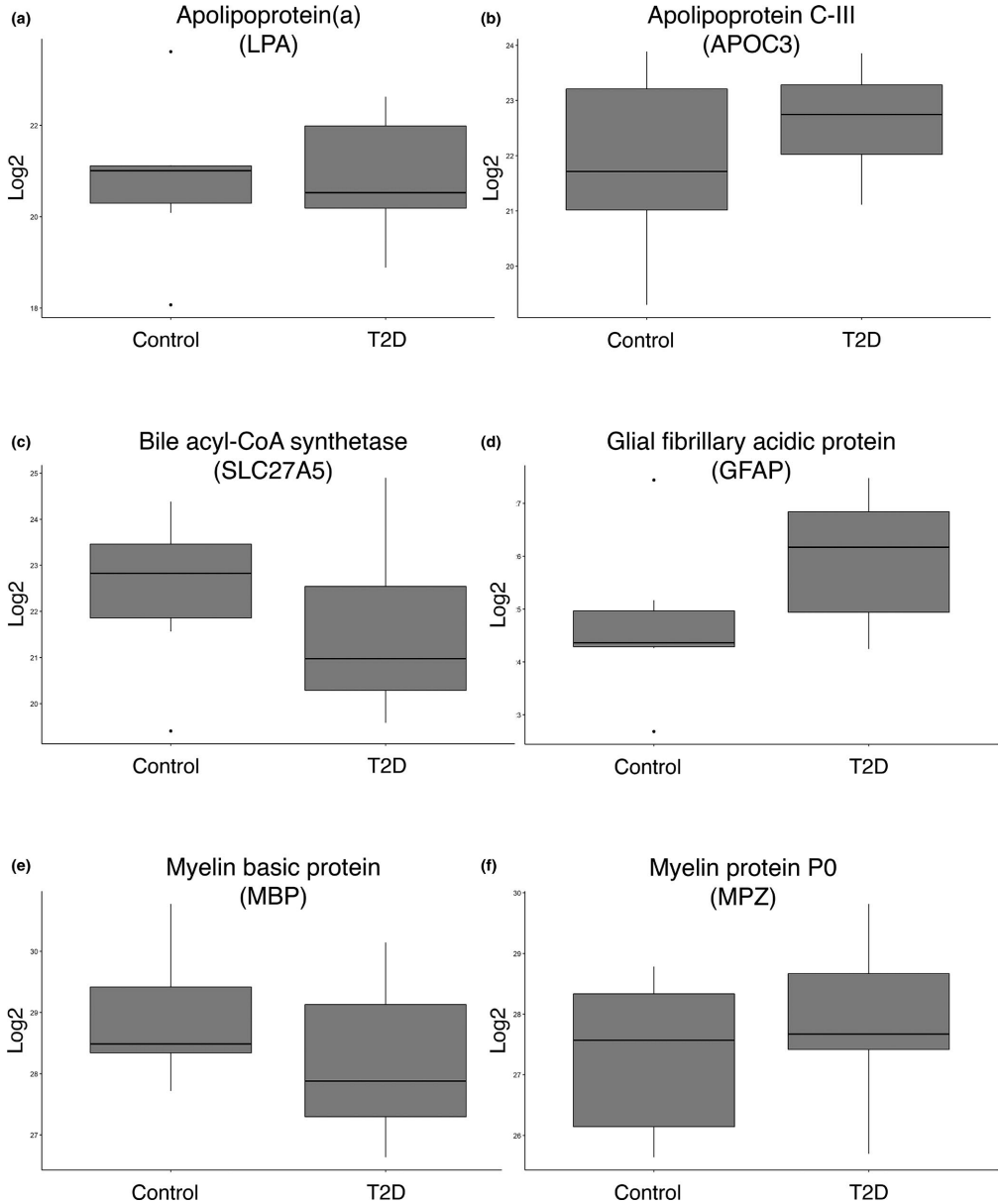

FIGURE 5 Boxplots of protein intensities for proteins associated with lipid metabolism and proteins associated with peripheral nerves. (a) Apolipoprotein(a) (LPA), (b) apolipoprotein C-III (APOC3), (c) bile acyl-CoA synthetase (SLC27A5), (d) glial fibrillary acidic protein (GFAP), (e) myelin basic protein (MBP) and (f) myelin protein P0 (MPZ). Boxplots show IQR of protein intensities with whiskers at 1.5× IQR and outliers ≤/≥ 1.5 × IQR

be involved in the analyses even if blood is 'washed away' from the nerves post-surgery. One must also consider that the variability of proteins expressed is great between individuals and is not solely connected to being diagnosed with type 2 diabetes or not. Furthermore, using proteomics does not provide a topographic location of where the proteins are active in the tissue. However, we strongly believe that providing a register of identified and quantified proteins will be helpful for further research on specific proteins.

The previously published electrophysiology data presented above state that there is a significant difference in SAMP among type 2 diabetes subjects compared to control, indicating at least early signs of DPN in the type 2 diabetes subjects, with lowered amplitudes of nerve conduction, indicating loss of nerve fibres. However, the use of PIN biopsies may be considered a possible limitation, since the PIN is located in the upper extremity, usually not as frequently affected by neuropathy as the lower extremity. Although the PIN is located distally in the upper extremity, it is not situated as peripheral as the sural nerve in the lower extremity, which is the conventional nerve for biopsy in clinical trials. Therefore, the PIN might not be as affected as the sural nerve, since DPN is length-dependent, affecting the most distal parts of the nerves first. Since the type 2 diabetes subjects only show early signs of DPN based on the neurophysiology examination of the sural nerve, and no differences in PIN morphometrics were seen between type 2 diabetes subjects and controls, one may consider that subjects with more widespread neurophysiological changes in the nerves of the upper extremity would express more extensive alterations in the neuronal proteome.

Although all participants in the study are close to of the same age, there is a significant difference in age between groups with the controls being slightly younger. We strongly believe that this age difference, of in median 2 years, will not impact the results in a cohort of participants around 70 years of age. A study by Ekman et al. on normative values of vibration perception thresholds shows that the thresholds deteriorate every year, as is expected considering the impact of ageing on neuronal health.[37] However, Ekman et al. show that the deterioration is small with only one year of aging, in both upper and lower extremities, while clearer changes in vibration perception thresholds can be seen over a longer aging period, for example 10 years.[37] Moreover, in this study, the nerve function of the upper extremity, analysed in the ulnar nerve not affected by any nerve compression lesion, did not differ between type 2 diabetes and healthy controls. Therefore, we believe that the early signs of DPN in the lower extremity, with lowered sSAMP among subjects with type 2 diabetes is more likely to be dependent on type 2 diabetes than age, which is in accordance with the fact that nerves in the lower extremities are more likely to develop DPN.[1]

In conclusion, we identified 2617 proteins in PIN from healthy participants and participants with type 2 diabetes. However, we did not see any clear differences in protein expression between groups significant enough to point out specific proteins of interest to DPN development. Possible reasons are the small study groups that morphometrics of PIN did not differ between healthy and type 2 diabetes participants and that the type 2 diabetes participants showed only early signs of DPN in the lower extremity and had normal electrophysiological values in the ulnar nerve of the upper extremity. Nevertheless, we have documented that using mass spectrometry analysis on human peripheral nerve tissue makes it possible to detect a wide range of proteins known to contribute to nerve damage and repair making them of interest to future studies on biomarkers for DPN.

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CONFLICT OF INTEREST

The authors declare no conflict of interests. At the time of this study, EÅ was affiliated and employed by Lund University, but after the completion of data collection and manuscript drafting she is employed by Thermo Fisher.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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Peripheral neuropathy in type 1 and type 2 diabetes

This doctoral thesis presents two novel approaches to the study of diabetic peripheral neuropathy, a severe complication to type 1 and type 2 diabetes. Today, there is no effective treatment for diabetic peripheral neuropathy, but treatment is focused on early discovery and improved metabolic control of diabetes. I present that diabetic peripheral neuropathy can be screened for in children and adolescents with type 1 diabetes using multi-frequency vibrometry. Furthermore, I describe the protein expression in the posterior interosseous nerve of the forearm, from subjects with type 1 and type 2 diabetes and subjects without diabetes, using quantitative proteomics.

Erik Ising started his PhD studies during his last semester of medical school at Lund University, and is currently working as a resident in emergency medicine at Skåne University Hospital in Malmö.

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